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5-FLUOROURACIL AS A PROBE FOR THE STUDY OF DIFFERENTIATION IN *DROSOPHILA MELANOGASTER*

by
Santhadevi Jeyabalan

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Zoology)
in The University of Michigan
1976

Doctoral Committee

Professor Tahir M. Rizki, Chairman
Professor Morris Foster
Professor Norman E. Kemp
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To my parents

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TABLE OF CONTENTS

	Page
DEDICATION	ii
ACKNOWLEDGEMENTS	iii
LIST OF TABLES	v
LIST OF ILLUSTRATIONS	vi
LIST OF APPENDICES	viii
LIST OF ABBREVIATIONS	ix
INTRODUCTION	1
MATERIALS AND METHODS	7
RESULTS	14
DISCUSSION	79
APPENDICES	109
BIBLIOGRAPHY	113

LIST OF TABLES

Table		Page
1.	Effects of fluorouracil concentration on the development of 70 (± 1)-hour-old third instar larva at the end of fifth day of treatment . . .	18
2.	Effects of treatments with fluorouracil, thymidine and uridine on the development of 70 (±1)-hour-old larva	24
3.	Effects of fluorouracil and thymidine on the development of third instar larva of different ages	26
4.	Effects of combined fluorouracil and thymidine treatment for different durations	34
5.	Effects of SDS and 2-mercaptoethanol on protein bands of third instar larval homogenate in SDS acrylamide electrophoresis	40
6.	Effects of different larval tissues on the protein bands of pupal extract in SDS acrylamide gel electrophoresis	44

LIST OF ILLUSTRATIONS

Figure		Page
1.	The effect of fluorouracil concentration on the development of third instar larva of *Drosophila*, at the end of fifth day of treatment	20
2.	The effect of fluorouracil concentration on the viability of larvae on the seventh day of treatment	23
3.	The effect of fluorouracil and thymidine on the development of third instar larvae of *Drosophila*, starting the treatment at different ages . . .	28
4.	The effect of fluorouracil and thymidine on the survival of larvae after seven days following initiation of treatment at different ages during the third instar stage	30
5.	The effect of fluorouracil and thymidine on protein content per larva	33
6.	The effect of SDS and/or 2-mercaptoethanol in the sample on the protein bands in SDS 10% acrylamide gel electrophoresis	39
7.	The effect of fluorouracil and thymidine treatment on the general protein banding pattern of *Drosophila* in SDS 10% acrylamide gel electrophoresis	48
8.	Schematic representation of gel containing all protein bands encountered during developmental stages of *Drosophila*, from 65-hour-old larva through 4-day-old pupa	49
9.	Log of molecular weight versus mobility of five standard proteins of known molecular weights in SDS 10% acrylamide gel	51
10.	The effect of fluorouracil and thymidine treatment on isozyme pattern during *Drosophila* development, (α- and β- esterases)	55

Figure | Page

11. The effect of fluorouracil and thymidine treatment on the isozyme pattern of Drosophila during development (acid and alkaline phosphatases) 58

12. A diagrammatic view of the organization of internal larval anatomy 61

13. The effect of FU + TdR on the development of proteinaceous globules in the fat body of Drosophila larva 66

14. The effect of FU + TdR on the fat body and wing imaginal disc of Drosophila larva 68

15. The effect of FU + TdR treatment on the salivary gland of Drosophila larva 72

16. The effect of FU + TdR on the differentiation of testis in Drosophila larva 75

17. Metabolism of FU and its sites of inhibition . . 82

LIST OF APPENDICES

	Page
Appendix I.	109
Appendix II.	110
Appendix III.	111
Appendix IV.	112

LIST OF ABBREVIATIONS

BIS:	N, N′ methylenebisacrylamide
BUdr:	5-bromodeoxyuridine
DNA:	deoxyribonueleic acid
dFUMP:	5-fluorodeoxyuridine monophosphate
dUMP:	deoxyuridine monophosphate, uridylate
EDTA:	ethylenediaminetetraacetic acid
FU:	5-fluorouracil
FUdR:	5-fluorodeoxyuridine
FUR:	5-fluorouridine
IUdR:	5-iododeoxyuridine
RNA:	ribonucleic acid
SDS:	sodium dodecyl sulfate
T:	thymine
TdR:	thymidine
TDP:	thymidine diphosphate
TMP:	thymidine monophosphate, thymidylate
TTP:	thymidine triphosphate
TEMED:	N, N, N′, N′ tetramethylethylenediamine
Tris:	trishydroxymethylamino methane
U:	uracil
UR:	uridine
UdR:	deoxyuridine

INTRODUCTION

Teratology, the study of congenital and induced developmental abnormalities, provides insight for understanding gene regulation and normal developmental processes and is an important aspect of pharmacological studies on the effects of various drugs on development. Many different teratogens, both physical and chemical agents, have been shown to produce "phenocopies" of genetic defects in different organisms including Drosophila (Landauer, 1957; Goldschmidt, 1957; Rapoport, 1939; Sang and McDonald, 1954; Rizki, 1960). Although some phenocopies imitate the final effects of mutation at the morphological level, the underlying biochemical mechanisms may not be the same.

Landauer (1957) has demonstrated that there are different critical periods of development when a phenocopying agent may be effective in producing different types of abnormalities. Thus, in the chick, at an early stage of development, insulin produces the phenocopy of rumplessness, whereas at a later stage it causes the phenocopy of shortened upper beak. These phenocritical periods are reflective of times when specific primordia related to axial skeleton or backbone are undergoing differentiation.

Knowledge of the mechanism of action of a phenocopying agent involves determining the biochemical pathways leading to structural modifications. Use of specific antimetabolites as phenocopying agents is therefore desirable. Most antimetabolites inhibit specific enzymes and thereby cause a number of secondary effects on overall development. Such an antimetabolite is the pyrimidine base analog, 5-fluorouracil (5-FU), first synthesized by Dutschinski and Heidelberger (1957) and shown to have carcinostatic activity by Heidelberger et al. (1957, 1958).

The effects of 5-FU have been studied in a variety of biological systems including microorganisms and mammalian cells in vitro. It has also been studied as a chemotherapeutic agent for human cancer (Heidelberger, 1965; Mandel, 1969; Burman, 1970). In mammalian and bacterial systems, 5-FU is converted to deoxyfluorouridine monophosphate (dFUMP) which acts as a specific inhibitor of the enzyme thymidylate synthetase and thereby blocks the conversion of deoxyuridine monophosphate (dUMP) to thymidine monophosphate (TMP). As a result of this block, the thymidine triphosphate (TTP) pool is depleted and DNA is no longer synthesized (Cohen et al., 1958; Heidelberger et al., 1960). Five-FU also acts as an analog of uracil and incorporates into RNA in place of uracil. The RNA containing fraudulent base may cause aberrations at the level of translation, i.e., in protein synthesis, and may

affect the structure of ribosomes (Bussard et al., 1960; Gross and Naono, 1961; Champe and Benzer, 1962).

The effects of 5-FU on the development of various organisms have been studied (Karnofsky and Basch, 1960; Tencer, 1961; Muth, 1965; Imberski, 1967; Rizki, 1968). When embryos of the sand dollar (Karnofsky and Basch, 1960) and Xenopus (Tencer, 1961) were treated with 5-FU or FUdR soon after fertilization, development was blocked at an early blastula stage. The inhibitory effect was prevented by thymidine but not by uridine. Since DNA synthesis and cell division are major events during early stages of these embryos, while very little change in RNA metabolism occurs, Karnofsky and Basch (1960) and Tencer (1961) suggested that the effect of 5-FU during early embryonic development might be at the level of DNA synthesis.

The teratogenic effect of 5-FU on later developmental stages has been studied in the meal moth Ephestia and in Drosophila (Muth, 1965; Imberski, 1967; Rizki, 1968). By implanting a crystal of 5-FU inside the pupal case of Ephestia, Imberski (1967) has shown that although normal differentiation of ommatidia occurred after treatment begun at a late pupal stage, treatment started at various intervals during early stages of pupation selectively inhibited the differentiation of different elements of ommatidia. Five-FU treatment of pupae at later stages prior to ommatidium differentiation did not inhibit its

final differentiation. Based upon these studies, Imberski (1967) concluded that (1) 5-FU affected the early determinative processes leading to differentiation, and (2) determination of different elements of ommatidia took place at different times during development. He also suggested that since little cell division took place in the eye disc of Ephestia after pupation, the inhibitory effect of 5-FU might be on the synthesis of RNA and not on DNA.

In Drosophila, Rizki (1968) has shown strain specific inhibition of facet formation in the eye and of the positioning of sense organs on the anterior cross vein of wing when third instar larvae were fed 5-FU for eight hours and then returned to their normal diet. He has further shown that an increase in the time for 5-FU feeding arrested development completely and prevented pupariation from occurring, although the larvae lived as long as ten to fourteen days. During this time larval cells regressed in size, as was obvious in the fat body cells due to the decrease in the cytoplasmic contents. However, the imaginal discs of the 5-FU-treated larvae retained the capacity to undergo metamorphosis when implanted into normal larva (Rizki, 1968). Rizki's pulse chase studies with ^{3}H - 5-FU on salivary glands of Drosophila in vitro showed that 5-FU first appeared in nucleolar and chromosomal RNA, but during a 25-minute chase, the label moved from nucleus to cytoplasm (Rizki, 1968).

Hence, the inhibitory effects of 5-FU on the development of Drosophila may be partly due to its incorporation into RNA.

Since information transfer from DNA to RNA very likely plays a major role in determination as well as in differentiation, Rizki's work (1968) indicating incorporation of 5-FU into RNA suggests that this compound might be utilized to interfere with DNA information transfer and thus provide insight into the control of gene expression in development. By interfering with RNA metabolism at different stages of development and then following morphological and biochemical processes in development, one can ask a question as to whether gene expression was affected at the time of treatment or whether a certain amount of autonomy exists, allowing gene products to be expressed in spite of prior interference.

One method of studying gene activity during development is to study qualitative and quantitative differences in gene products, proteins and isozymes at different developmental stages. In Drosophila, stage specific changes in the electrophoretic pattern of general proteins and certain isozymes are known to occur, indicating that larval and adult development involve expression of different genes (Wright, 1963; Johnson, 1966; Duke and Pantelorius, 1967; Pasteur and Kastristis, 1971). Since it is already known that in Drosophila 5-FU administration

to the larva prevents pupariation and subsequent development (Rizki, 1968), although the larva continues to live for more than ten days, the question arises whether blocking the larva from entering pupariation affects the elaboration of pupal specific proteins.

The present study is aimed at answering the following specific questions. What are the most desirable conditions for blocking the progression from larval to pupal form? What is the critical stage of development for initiating the blocking by treatment? What concentration of 5-FU is most effective for blocking further development but least harmful to survival of the larva? How and to what extent can the 5-FU-affected larva recover from the developmental block? How far do the different tissues undergo differentiation after 5-FU treatment? Finally, are the pupal protein patterns intimately coupled with development and differentiation, or can they be uncoupled by 5-FU so that electrophoretic patterns and stage of development are not intimately linked?

MATERIALS AND METHODS

Collection and timing of larvae

An Ore-R wild type strain of *Drosophila melanogaster* was used in the present study. Eggs and larvae were collected on spoons containing honey and yeast-coated cream of wheat medium according to the method described by Rizki (1960). Larvae were aged ± one hour from the time of eclosion of eggs. To avoid overcrowding and to provide optimal growth conditions, about a hundred larvae were picked up from spoons and transferred to a crystalline dish, 10 cm in diameter X 5 cm deep, containing cream of wheat medium seeded with Fleischman's yeast.

The first instar stage lasts for 24 hours, the second instar for 24 hours and the third instar for 48 hours, which is followed by puparium formation and the pupal stage extending over a period of four days.

When the larvae reached the desired age, they were collected by floating them in saturated sodium chloride solution. They were washed once with 1% Clorox to remove external contaminants and rinsed several times in distilled water to wash off Clorox before experimental treatments were begun.

Treatments

Thirty larvae were introduced into glass petri dishes 60 mm in diameter, containing washed Whatman paper pulp

saturated with test solution. The test solutions used were 5-FU, uridine, thymidine, and mixtures of them, dissolved in glass-distilled water. In order to prevent the evaporation of test solutions and escape of larvae, the tops of petri dishes were lined with folded Kleenex paper tissue. The paper pulp was kept moist by the addition of appropriate test solution when necessary. All test solutions were made with glass-distilled water.

Histochemical studies

Larvae were fixed overnight in Carnoy's fixative, three parts 85% ethanol to one part glacial acetic acid, prepared just before use. They were embedded in paraffin (melting point 56-58°C) and longitudinal, serial sections of 5 μ thickness were cut.

The protein staining procedure of Mazia et al. (1953) was followed. After deparaffinization and rehydration, the sections were stained for 15 min at room temperature in a solution containing 10 gm of mercuric chloride and 100 mg of bromphenol blue in 100 ml distilled water. After staining, the sections were washed in 0.5% acetic acid for 20 min and then immersed for 3 min in distilled water adjusted to pH 6.5 with 0.1 N NaOH. Dehydration was carried out starting with 70% ethanol. The sections were cleared in xylene and mounted in Permount. Proteins were stained dark blue by this procedure.

Homogenization

Thirty to fifty larvae or pupae were homogenized on ice by ten up-and-down strokes with a Potter-Elvehejm all-glass homogenizer in 1 ml of buffer consisting of 0.0625 M Tris, 0.01 M 2-mercaptoethanol and 0.001 M EDTA, adjusted to pH 6.8. The homogenization buffer also contained a few crystals of phenylthiourea to prevent tyrosinase activity. Five-tenths ml of this crude homogenate was used for isozyme studies. To the remaining 0.5 ml of the homogenate, 0.5 ml of a solution consisting of 2% SDS, 2% 2-mercapto-ethanol,1% glycerol, and 0.005% bromphenol blue in 0.0625 M Tris buffer at pH 6.8, was added. The mixture was immediately placed in a boiling water bath and boiled for 15 min. The undissolved materials were centrifuged away, at 3000 rpm for 3 min in a clinical centrifuge and the supernatant was stored in the refrigerator for later analysis of proteins by SDS acrylamide electrophoresis.

Protein concentration was measured by the method of Lowry et al. (1951), using a Coleman Junior spectro-photometer. Crystalline bovine serum albumin was used as a standard.

Electrophoresis

Electrophoresis in 10% acrylamide gels containing SDS was based on Laemmli's method (1970) with modification for the present study. The stock solutions are listed in appendix II. The lower or separating gel consisted of 10% (W/V) acrylamide, 0.27% (W/V) bis., 0.025% (V/V)

TEMED, and 0.033% (W/V) ammonium persulfate in 0.125 M Tris-HCl buffer, pH 8.8. The lower gel mixture was introduced into borosilicate tubes, 10 cm long and 6 mm inner diameter, to the height of 8 cm, and was overlain with distilled water to the height of 0.5 cm. Chemical polymerization of the lower gel was complete within 45 min. The top water layer was then shaken out and the top of the lower gel was rinsed with the stacking or upper gel mixture. The final concentrations of the different ingredients in the upper gel were 3% acrylamide, 0.09% bis., 0.1% SDS, 0.025% TEMED and 0.033% ammonium persulfate in 0.0625 M Tris-HCl buffer, pH 6.6. The upper gel mixture was introduced on top of the polymerized lower gel to the height of 1.0 cm and was overlain with distilled water. The polymerization of upper gel was complete in 45 min to 1 hr. The water was shaken out and the gel tubes were fitted in the upper bath assembly of the electrophoresis apparatus (distributed by Buchler Instruments); then 225 ml of electrode buffer was introduced into the lower bath and 400 ml into the upper bath. The electrode buffer was made up of 0.025 M Tris, pH 8.3, 0.192 M glycine and 0.1% SDS.

Electrophoretic study of proteins

For the study of electrophoretic pattern of general proteins, the sample was prepared as explained under Homogenization. Sample solutions were brought to room temperature and a volume containing 30 µg protein was layered directly over the stacking gel. The volumes of

all the samples used for a single run were kept constant by adding an appropriate volume of homogenization buffer to reach the volume of the least concentrated sample. The range of sample volume in different runs was 100 - 150 ul.

Electrophoresis was carried out at room temperature at five milliamperes per gel, using a Canalco model 1400 electrophoresis power supply with constant rate source. When the tracking dye (bromphenol blue) reached 0.5 cm from the bottom of the gel, electrophoresis was stopped. The gels were taken out of the tubes and stained in 0.05% Coomassie blue in 9% (V/V) glacial acetic acid and 50% methanol for 2-3 hr at room temperature. Gels were next destained by diffusion in 7½% acetic acid and 5% methanol in distilled water and were stored in 7½% acetic acid. The gels were scanned at 580 nm with a Gilford densitometer.

Electrophoretic study of isozymes

For isozyme studies, 6% acrylamide gels without SDS were used. The samples were prepared as described under Homogenization. The procedure for electrophoresis was the same as that for general proteins except for the following:

1. The height of the separating gel was 5 cm.
2. The electrode buffer did not include SDS and was refrigerated prior to use.
3. The samples were kept on ice until application on gels.

4. The electrophoresis was carried out at room temperature with cold tap water running through the jacket around the lower buffer compartment to avoid excessive heat that might be produced during electrophoresis.
5. The gels were run at two milliamperes per gel.
6. Sixty to 100 ug of protein were applied on each gel.

For staining esterases, the gels were preincubated for 20 min in 0.05 M sodium acetate buffer, pH 5.0. They were then transferred to a solution containing 10 mg fast GBC garnet salt and (for α-esterases) 1 ml of 1% α-naphthyl acetate in water; acetone (1:1 volume), or (for β-esterases) 1 ml of 1% β-naphthyl acetate in pure acetone, per 10 ml 0.05 M sodium acetate buffer, and were incubated for 30 min.

Gels were stained for acid phosphatase by preincubating the gels in 0.05 M acetate buffer, pH 5.0, for 20 min and then transferring them to a solution containing 10 mg α-naphthyl phosphate and 10 mg fast GBC garnet salt per 10 ml 0.05 M acetate buffer, pH 5.0, for 30 min. For alkaline phosphatases, the gels were incubated for 30 min in a solution containing 10 mg α-naphthyl phosphate, 10 mg fast red TR in 10 ml 0.1 M tris-HCl buffer, pH 9.5, and rinsed several times in distilled water.

At the end of incubation period, all the gels were rinsed in distilled water and stored in 7½% acetic acid. For all the four isozymes mentioned above, appropriate controls were run by omitting the substrate in the incubation mixture.

RESULTS

Normal development of pupa

A brief account of normal developmental changes in the external morphology of *Drosophila melanogaster* during the period of interest (larval-pupal transformation) is as follows: According to Bodenstein (1950), at the end of the third larval instar, shortly before puparium formation, the larva of *Drosophila* ceases to feed and becomes quiescent. The anterior spiracles are everted and within a short time the larva shortens, probably due to muscular contraction. Larval segmentation is obliterated externally and the larval cuticle becomes the "puparium". The white puparium hardens and darkens, and about 3½ hr later the puparium is fully tanned. About 2 hours after the puparium is formed, an operculum develops by flattening of the anterior dorsal surface of the puparium. About 4 hours after quiescence, a prepupal molt occurs which involves the separation of internal tissues from the puparium except at the spiracles, mouth parts and hind gut lining. The pupal molt that occurs about 12 hours after puparium formation involves the shedding of the prepupal cuticle and the separation from the anterior and posterior connections. The imaginal discs are everted and a typical pupa with head, thorax and abdomen is formed.

Gross effects of fluorouracil treatment

Fluorouracil treatment of *Drosophila* larvae gave rise to an array of developmental abnormalities. Arbitrarily, the FU-induced syndromes are described according to decreasing intensity of disturbances and are grouped into the following four categories:

L. A permanent larva. Puparium formation and subsequent development were completely blocked. The larva lived for about 10 to 12 days after the treatment started and then died.

Ltc. A larva in which tanning and hardening of larval cuticle took place, but contraction of cuticle and operculum formation failed to occur. In some cases the spiracles were everted. Partial pupal molting of the posterior region occurred as evidenced by separation of the tissues from the cuticle, leaving empty space at the posteriormost region.

P. Abnormal pupa. Shortening of the larva in length and puparium formation occurred. Anterior spiracles were everted. The cuticle became hardened and tanned, and the operculum was formed. After this, further developmental processes were arrested at different levels and are grouped under three sub-categories:

P_1. Tissues in the posterior region were detached from the puparium, whereas mouth parts and tracheae were still attached to the puparium at the anterior region.

P_2. Pupal molting within the puparium occurred, but head eversion and shaping of head, thorax and abdomen never proceeded.

P_3. Delineation of head, thorax and abdomen occurred, but eye pigments were never developed.

Np. Normal pupa. Normal looking adults with eye pigments developed within the puparium, but they died inside the pupal case. In some cases, a "ptilinium", the eversible sac located just above the base of the antennae, was formed. The operculum was opened up, but the adults died at that point.

Effect of concentration of fluorouracil

In all the feeding experiments, it was observed that the digestive tract of larvae contained paper pulp. Since paper pulp was saturated with test solution, it was possible that the larva ingested test solution along with paper pulp. It was also possible that the test solution diffused in via larval cuticle.

In order to study the effects of different concentrations of fluorouracil on external developmental changes in Drosophila melanogaster and to choose a suitable dose which would have maximal effect in arresting puparium

formation and subsequent development but a minimal effect on mortality, 70 (±1)-hour-old larvae were continuously treated with different concentrations of FU. The highest concentration used was 4.8 mg FU/ml. From this, one-to-one serial dilutions were made, and the lowest concentration used had 4.7 ug/ml. Control groups of larvae were treated in distilled water. Observations were made daily. The results, summarized in Table 1 and Figure 1, are based on observations on the fifth day after treatment started. Larvae in the control group had completed development and emerged as adults on the fifth day of treatment.

At low concentrations of FU, 18.8 µg or below, development was not blocked; hence 87% or more of larvae emerged as adults. Higher concentrations of FU, 150 µg and above, were effective in blocking puparium formation in 93% or more of the larvae. At concentrations of 37.5 µg and 75 µg/ml of FU, development was blocked at various levels, as shown by the occurrence of all four categories of abnormal syndromes (Table 1). The control showed approximately 98% normal pupation and adult emergence. For the production of 50% permanent larvae, the effective dose, ED 50, is between concentrations of 50-70 µg FU/ml (Figure 1).

In this series, after six days of treatment, the viability of larvae began to show a significant decline. The method of deciding whether a larva was dead or alive

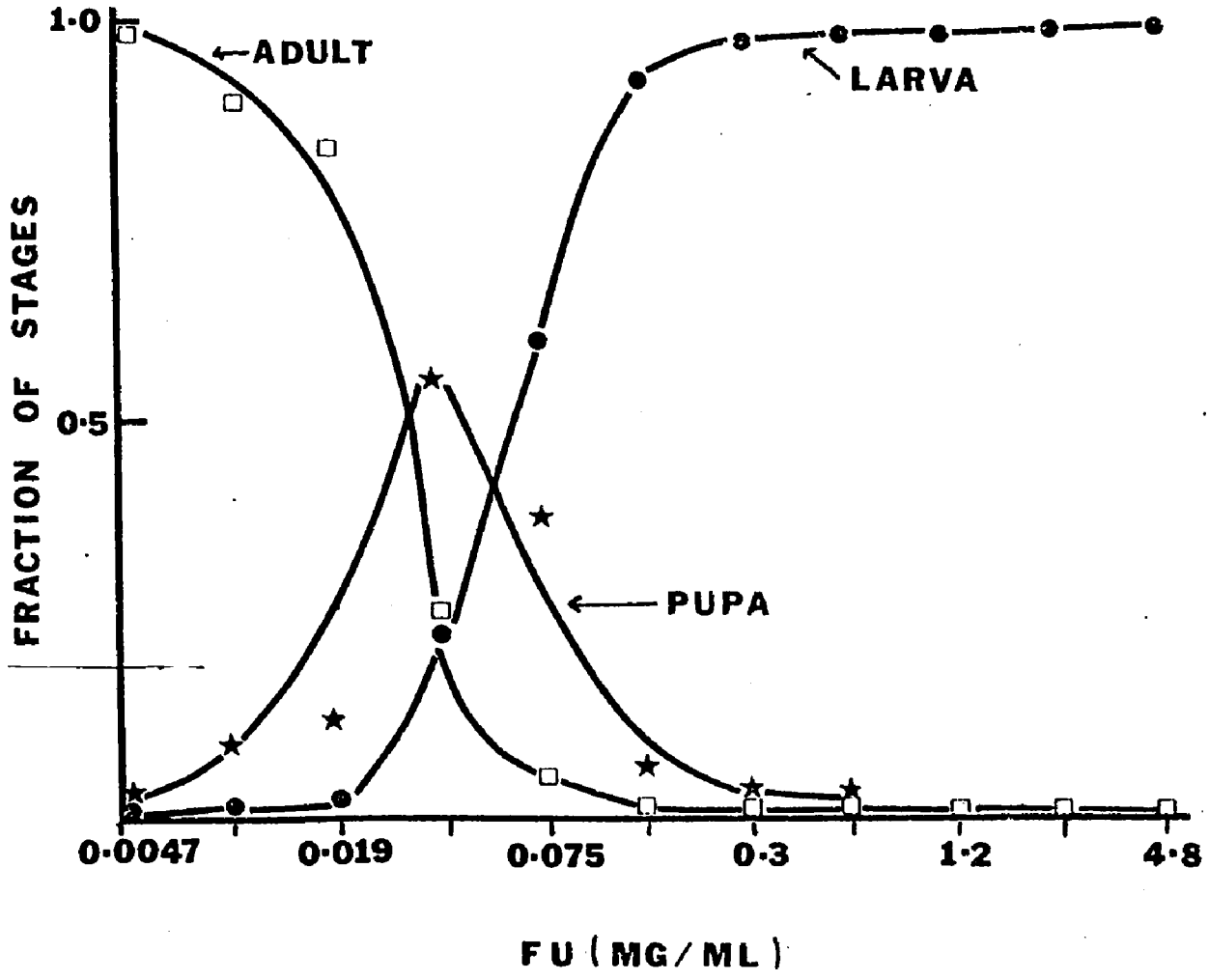
Table 1. Effects of fluorouracil concentration on the development of 70 (± 1)-hour-old third instar larvae at the end of fifth day of treatment.

FU conc. (µg/ml)	Permanent larvae	Larvae tanned	Abnormal pupae	Normal pupae	Adults	N
4.7	0	0	0.011±0.019[1]	0	0.989±0.019	90
9.4	0	0	0.067±0.057	0.022±0.019	0.911±0.084	90
18.8	0	0	0.056±0.051	0.067±0.033	0.877±0.077	180
37.5	0.244±03.031	0.299±0.016	0.156±0.031	0.067±0.000	0.233±0.078	180
75	0.60 ±0.031	0.305±0.039	0.011±0.000	0.022±0.016	0.050±0.017	180
150	0.933±0.066	0.053±0.068	0.013±0.021	0	0	180
300	0.986±0.025	0	0.014±0.024	0	0	180
600	0.989±0.019	0	0.010±0.019	0	0	90
1200	0.989±0.019	0	0	0	0	90
2400	1.000±0.0	0	0	0	0	90
4800	1.000±0.0	0	0	0	0	90
H20 cont.	0	0	0.006±0.003	0.011±0.03	0.983±0.078	180

[1]Fraction affected ± S.D.

Figure 1. The effect of fluorouracil concentration on the development of third instar larvae of Drosophila, at the end of fifth day of treatment. The larvae were 70 (± 1) hours old when treatment started. Each plotted value represents mean of at least three replicates of 30 larvae.

FIGURE 1


1·0
0·5
FRACTION OF STAGES
ADULT
LARVA
PUPA
0·0047
0·019
0·075
0·3
1·2
4·8
FU (MG/ML)

was simply to determine whether it was responsive to mechanical stimuli. Figure 2 represents larval viability in different concentrations of FU on the seventh day of treatment. It is obvious from the graph that when concentration of FU was increased, the percent of larval viability decreased, reaching 18% at the concentration of 4.8 mg FU/ml. The dose at which 50% survival (SD 50) occurred was about 2.4 mg FU/ml.

The concentration chosen for further studies, 0.3 ml FU/ml, resulted in about 99% permanent larvae with no apparent mortality effect up to seven days of treatment.

Effects of treatment with fluorouracil and nucleosides

In order to determine whether uridine (UR) and/or thymidine (TdR) might overcome the adverse effects of 5-FU in Drosophila, 70 (± 1)-hour-old larvae were treated with these nucleic acid precursors alone, together or in combination with fluorouracil as listed in Table 2. Concentrations of TdR or UR were two times that of FU. Neither thymidine nor uridine fed in combination with FU modified the effect of FU in producing 100% permanent larvae. Control treatments with thymidine and/or uridine had no apparent effects on normal development since animals metamorphosed as readily as controls in water.

Effects of fluorouracil on larvae of different ages

Larvae of different ages during the third instar stage, namely, 65, 67, 72, 78, 81 and 83 (± 1)-hours old, were continuously treated in 0.3 mg FU and 1.2 mg TdR/ml.

Figure 2. The effect of fluorouracil concentration on the viability of larvae on the seventh day of treatment. Larvae were 70 ($\pm$ 1) hours old when treatment started. Each value represents the mean and standard deviation of at least three replicates of 30 larvae.

FIGURE 2

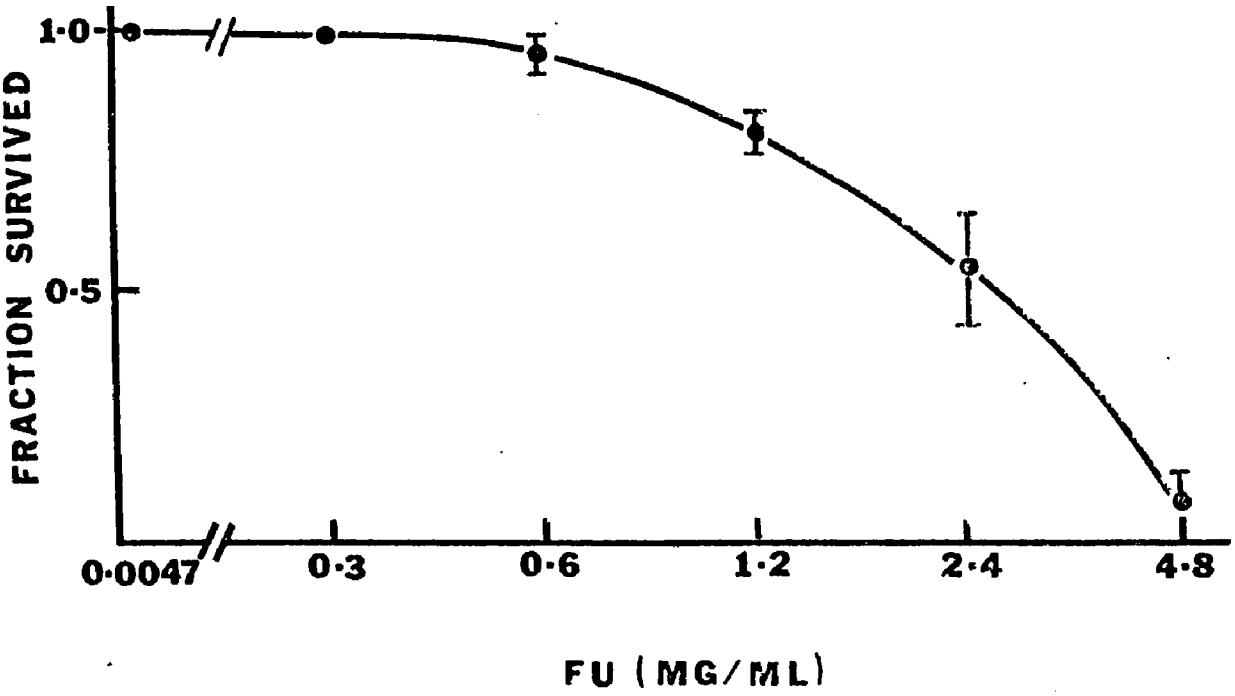


Table 2. Effects of treatments with fluorouracil, thymidine and uridine on the development of 70 (± 1)-hour-old larvae.

Treatment	Permanent larvae	Larvae tanned	Abnormal pupae	Normal pupae	Adults	N
FU	0.992±0.011[1]	0	0.008±0.012	0	0	120
FU+TdR	1.000±0.0	0	0	0	0	120
FU+TdR+UR[2]	1.000±0.0	0	0	0	0	120
TdR	0	0.025±0.032	0.025±0.017	0.025±0.032	0.925±0.063	120
UR	0	0	0.008±0.012	0.008±0.012	0.983±0.019	120
TdR+UR	0	0	0.008±0.012	0.008±0.012	0.983±0.063	120
H_2O	0	0	0.008±0.012	0.017±0.019	0.975±0.0	120

[1]Fraction affected ± S.D.

[2]Concentrations of chemical agents:

0.3 mg FU per ml.
1.2 mg TdR per ml.
1.2 mg UR per ml.

Water controls were established for each age studied. Very early third instar larval ages were avoided, since starvation effects would result in puparial arrest even in water controls.

The results are summarized in Table 3 and Figure 3. The FU effect of blocking puparium formation was complete if the treatment was started before the larvae were 78 hours old. Incomplete effects were noticed when the treatment was started after that age. Eighty-one-hour-old larvae were apparently affected variably, as indicated by the occurrence of all different categories of abnormalities (Table 3). Results on larvae of 83 hours were also variable but difficult to interpret because of the failure of larvae to remain on paper pulp.

Survival of larvae as a function of larval age at the time of treatment was determined by scoring the viable larvae on the seventh day of treatment. Results are presented in Figure 4. The earlier the treatment was started, the lower was the percentage of survival. About 85% of larvae survived on the seventh day when treatment was begun at 65 hours, compared with 98% survival when treatment began at 83 hours of age. Water controls by the seventh day had eclosed as adults.

Effect of fluorouracil on protein content of larvae

To study the effects of FU on the total protein content per larva, 70 (± 1)-hour-old larvae were continuously treated in 0.3 mg FU and 1.2 mg TdR/ml, and ten

Table 3. Effects of fluorouracil plus thymidine on the development of third instar larvae of different ages.

Age in hours (±1 hr)	Treatment	Permanent larvae	Larvae tanned	Abnormal pupae	Normal pupae	Adults	N
65	FU+TdR[1]	0.099±0.015[2]	0	0	0	0.007±0.015	150
65	H_2O	0	0	0.013±0.029	0	0.987±0.029	150
67	FU+TdR	0.987±0.029	0	0.013±0.029	0	0	150
67	H_2O	0.013±0.029	0	0.013±0.019	0.033±0.042	0.933±0.043	150
72	FU+TdR	0.979±0.028	0.003±0.011	0.013±0.023	0	0	300
72	H_2O	0.003±0.011	0	0.010±0.022	0.003±0.011	0.979±0.023	300
78	FU+TdR	0.959±0.028	0	0.039±0.027	0	0	150
78	H_2O	0	0	0	0.039±0.036	0.959±0.036	150
81	FU+TdR	0.374±0.126	0.033±0.047	0.325±0.185	0.149±0.694	0.142±0.216	120
81	H_2O	0	0	0	0.008±0.016	0.992±0.016	120
83	FU+TdR	0.083±0.019	0	0.525±0.119	0.217±0.088	0.149±0.064	120
83	H_2O	0	0	0	0	1.000±0.0	120

[1]Concentrations of chemical agents:

0.3 mg FU per ml.
1.2 mg TdR per ml.

[2]Fraction affected ± S.D.

Figure 3. Effect of fluorouracil and thymidine (0.3 mg FU, 1.2 mg TdR / ml) on the development of third instar larvae of Drosophila, starting the treatment at different ages. Each plotted value is the mean of at least four replicates of 30 larvae.

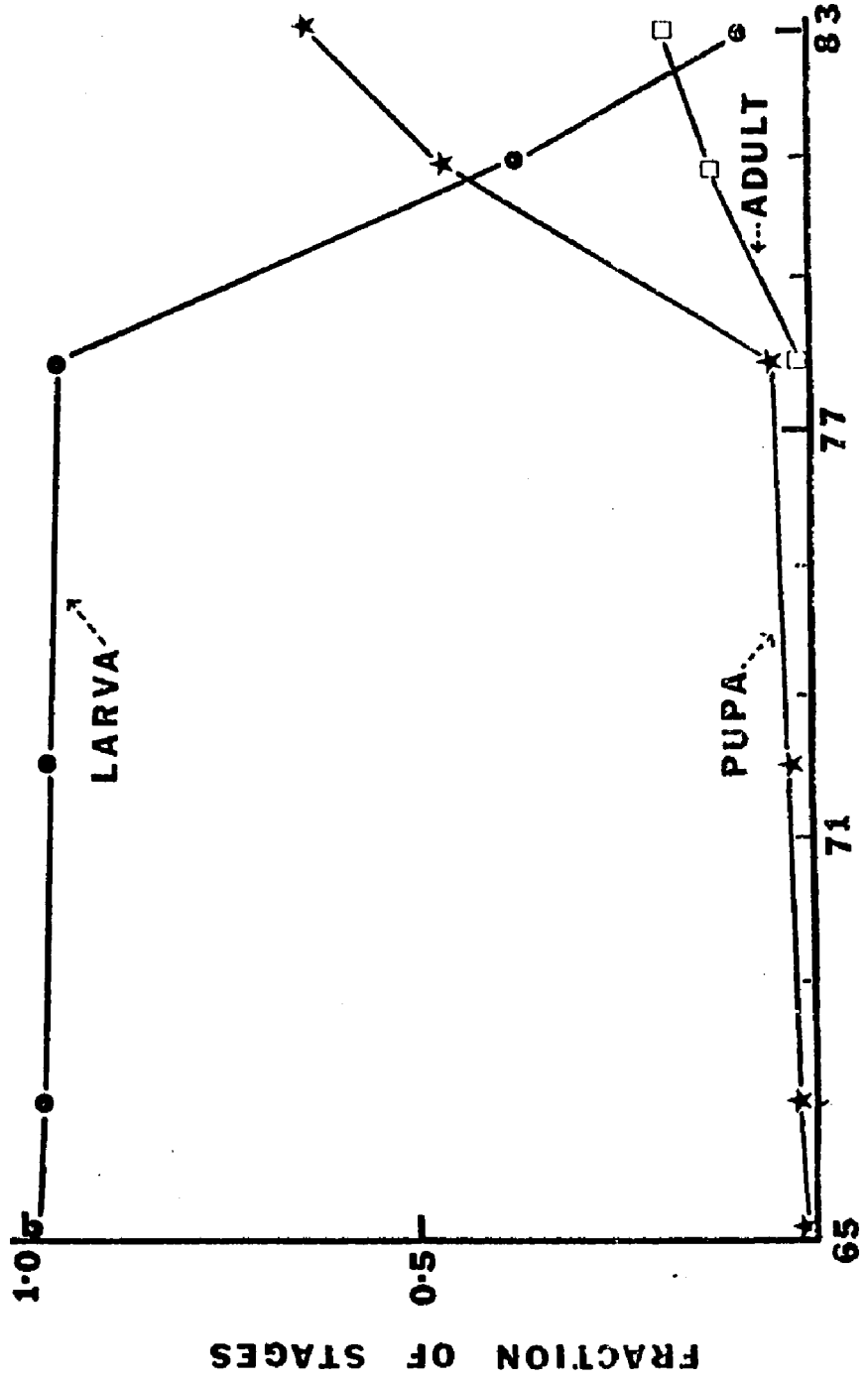
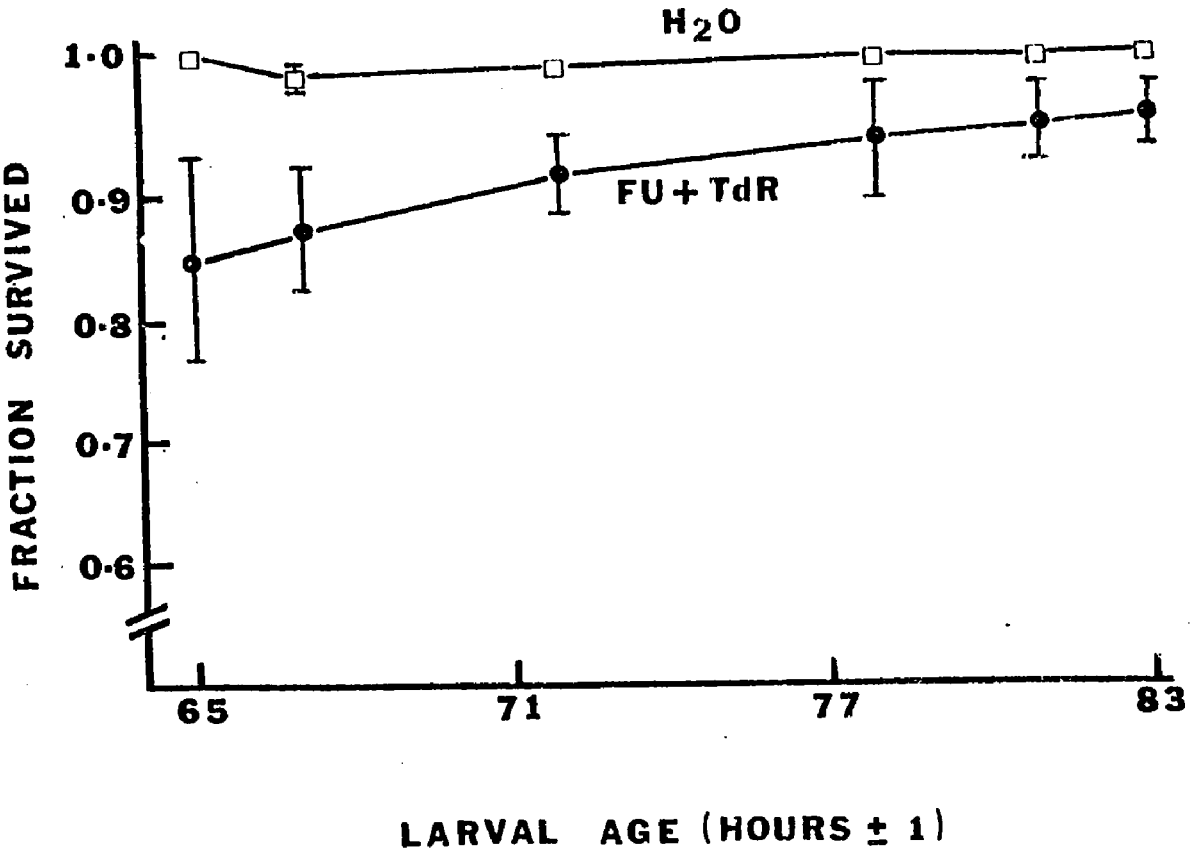

1·0
0·5
FRACTION OF STAGES
65
71
77
83
LARVAL AGE (HOURS ± 1)
LARVA
PUPA
ADULT

FIGURE 3

Figure 4. Effect of fluorouracil and thymidine (0.3 mg FU, 1.2 mg TdR / ml) on the survival of larvae after seven days following initiation of treatment at different ages during the third instar stage. Each plotted value is the mean and the bars, the standard deviation of at least four replicates of 30 larvae.

FIGURE 4


H2O
FU + TdR
1·0
0·9
0·8
0·7
0·6
FRACTION SURVIVED
65
71
77
83
LARVAL AGE (HOURS ± 1)

larvae from each of three different dishes similarly treated (FU-TdR, or water for controls) were homogenized in one ml of tris buffer. Four ml of distilled water was then added and protein concentration in 1 ml of the mixture was measured by Lowry's method. Protein content per larva was calculated.

Results are summarized in Figure 5. The protein concentration per larva decreased as FU treatment was continued. The decrease in protein content per individual was also observed in water controls when larvae were undergoing normal pupation. Hence, it appears that the decrease in protein content might be due to starvation effects.

Effect of fluorouracil treatment of variable duration

Some experiments were done to see whether recovery from adverse effects of FU occurred if larvae were treated for a certain duration and then transferred back to food or water. Seventy (± 1)-hour-old larvae were treated in 0.3 mg FU and 1.3 mg TdR/ml for 27, 50, 74 and 94 hours, washed well and transferred to bottles containing cornmeal molasses medium and to petri dishes containing paper pulp saturated with water. Observations were made at intervals of 24 hours. Results are summarized in Table 4.

Larvae treated for 50 hours or more in FU + TdR and transferred to water showed complete block of puparium formation. About 100% remained permanent larvae, indicating that FU effects were not reversed under these conditions.

Figure 5. Effect of fluorouracil and thymidine (0.3 mg FU, 1.2 mg TdR / ml) on protein content per larva. Larvae were 70 (± 1) hours old when treatment started. Each plotted value is the mean ± standard deviation of three replicates of samples of 10 larvae. In water controls, the larvae pupated by third day after treatment started and adults eclosed by seventh day.

FIGURE 5

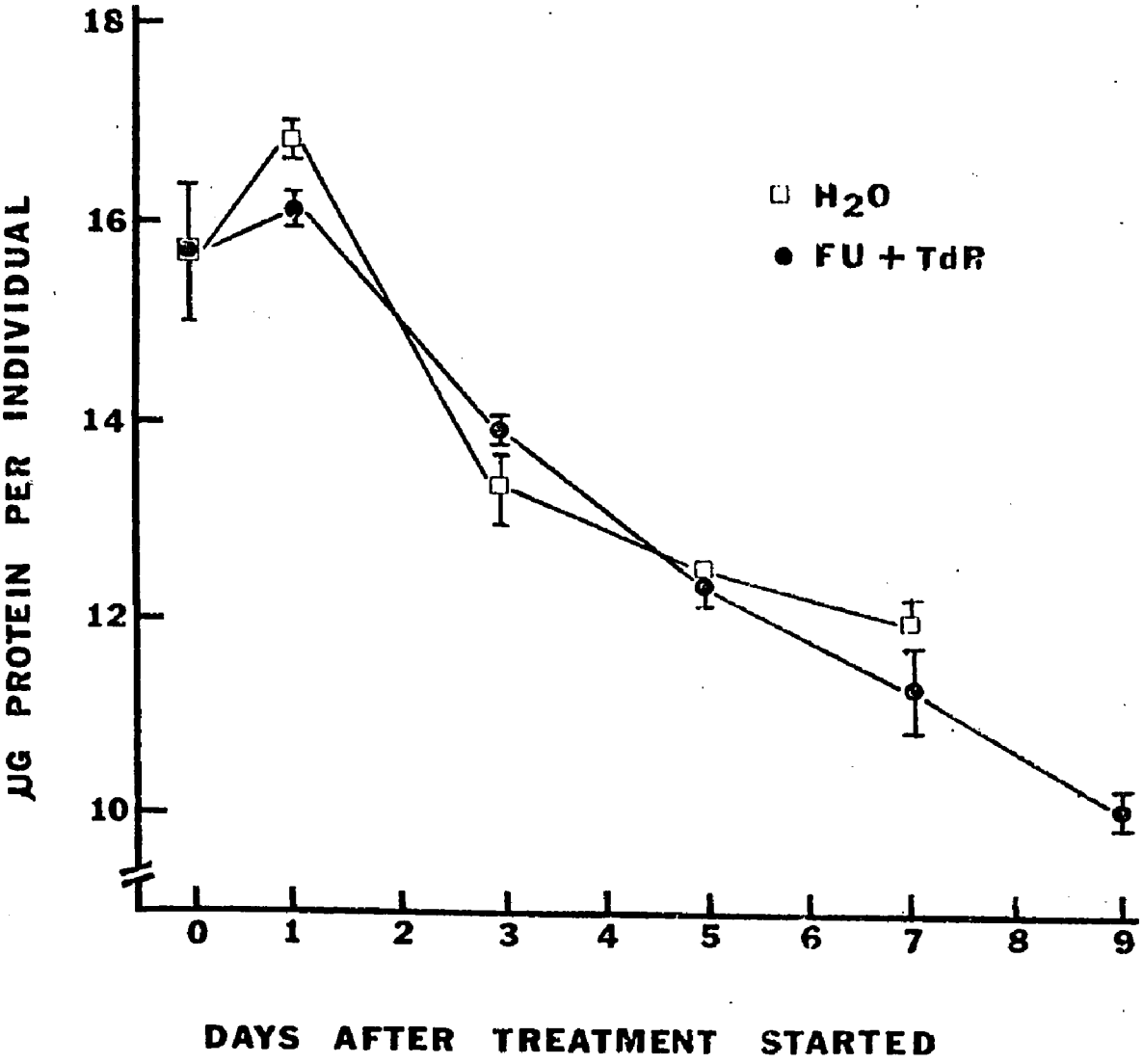

18
16
14
12
10
μG PROTEIN PER INDIVIDUAL
H2O
FU + TdR
0
1
2
3
4
5
6
7
8
9
DAYS AFTER TREATMENT STARTED

Table 4. Effects of combined fluorouracil and thymidine treatment for different durations.

Time of treatment in FU+TdR[1,2]	Transferred to	Permanent larvae	Abnormal pupae	Adults	N	Mean age at puparium formation (in hours)	Mean age at adult eclosion (in hours)
27 hr	food	0.04[3]	0.88	0.08	50	256±12	257±12
27 hr	water	0.86	0.14	0.00	50	167±12	
50 hr	food	0.14	0.86	0.00	100	244±12	
50 hr	water	0.99	0.01	0.00	100		
74 hr	food	0.31	0.69	0.00	100	395±12	
74 hr	water	1.00	0.00	0.00	100		
94 hr	food	0.65	0.35	0.00	100	488±12	
94 hr	water	1.00	0.00	0.00	100		
9 days[4]	-	1.00	0.00	0.00	100		
9 days in H_2O[4]	-	0.00	0.00	1.00	100	97±2	197±4

[1]Larvae 70 (±1)-hours-old when treatment started.
[2]Concentrations of chemical agents: 0.3 mg FU per ml plus 1.2 mg TdR per ml.
[3]Fraction affected.
[4]Continuous treatments without transfer.

Treatment in FU + TdR for 24 hours followed by return to water, however, resulted in 14% of larvae undergoing puparium formation. Transfer to food instead of water yielded different results. As is obvious from Table 4, puparium formation occurred in larvae treated in FU + TdR for as long as 94 hours before transfer to food. The longer the larvae were treated in FU + TdR and then transferred to food, the lesser the number of larvae that went through normal puparium formation. Furthermore, for those undergoing pupariation, the mean time at which puparium formation occurred was longer in larvae treated for longer periods in FU + TdR prior to transfer to food.

Electrophoresis of proteins

Larval and pupal homogenates

Since there were no apparent quantitative differences in total protein content between fluorouracil-blocked larvae and normal pupae, attention was directed toward a study of qualitative similarities and differences in the proteins of the samples. While standardizing the SDS acrylamide disc gel electrophoresis technique to study general proteins, inconsistent results were obtained when the samples were treated in slightly different ways prior to application on the gels. Effort to explain these findings led to the results reported in this section.

After homogenization of normal or fluorouracil-blocked larvae and pupae as described under Materials and Methods, an equal volume of 0.0625 M tris-HCl buffer,

pH 6.8, containing 2% SDS, 2% mercaptoethanol, 0.005% bromphenol blue and 10% glycerol, was added to the whole homogenate and the mixture was incubated at 37°C for one hour. Electrophoresis and subsequent staining for general proteins were carried out as described in Materials and Methods. Thirty (30) µg of proteins was loaded on each gel. Gels containing larval or FU-blocked larval homogenate showed no protein bands, whereas those containing pupal extract had numerous protein bands from the top of the gel to the tracking dye front.

Different results were observed when sample homogenates were not mixed with SDS nor with mercaptoethanol but were subjected to electrophoresis in non-SDS acrylamide gel as for isozyme study (see Materials and Methods) and stained with Coomassie blue. After this treatment both larval and pupal extracts yielded protein bands. Attention then turned toward an explanation for the absence of protein bands in SDS gels containing larval extracts.

To determine whether the incubation process prior to electrophoresis was affecting the sample protein content, the following experiment was devised. Larval extract mixed with various components of the usual incubation mixture was incubated for one hour at 37°C, 25°C or cooled with ice. Results of treatment with 1% SDS, 1% mercaptoethanol, 1% SDS and 1% mercaptoethanol or with buffer are summarized in Table 5 and Figure 6. Buffer treatment alone resulted in an absence of bands in the top

one third of the gel, where high molecular weight bands might be expected to band. Mercaptoethanol treatment gave the same results as buffer only. SDS treatment, however, resulted in the elimination of protein bands except in samples kept at 0°C such that no protein bands appeared on gels.

To test whether buffer and/or SDS might be activating enzymes responsible for reduced protein banding, larval extract with 1% SDS and 1% mercaptoethanol was kept in a boiling water bath for 5 - 10 minutes prior to electrophoresis. Enzymes presumably might be denatured by boiling. Gels after this treatment showed the full banding pattern from the top of the gel to the front (Figure 6,6). It appeared therefore that larval extracts might indeed contain proteolytic enzymes removable by boiling.

To check whether the disappearance of high molecular weight proteins in larval samples incubated with buffer or with buffer plus mercaptoethanol occurred during the incubation process, a sample was incubated at 37°C in buffer or mercaptoethanol for one hour and then boiled for 5-10 min in the presence of SDS. This procedure resulted in full protein banding of the gel. The same experiment after incubation of the sample with SDS, or with SDS plus mercaptoethanol resulted in no bands on the gels. The results of these experiments suggest that the absence of high molecular weight bands in samples incubated with

Figure 6. The effect of SDS and/or 2-mercaptoethanol in the sample on the protein bands in SDS 10% acrylamide gel electrophoresis.

1. Extract of third instar larva, incubated in 1% SDS and 1% mercaptoethanol at 37°C for one hour.
2. Extract of larvae treated with FU for 9 days, incubated in 1% SDS and 1% mercaptoethanol at 37°C for one hour.
3. Larval extract, kept in ice with 1% SDS and 1% mercaptoethanol for one hour.
4. Larval extract, incubated in 1% mercaptoethanol for one hour at 37°C.
5. Larval extract stored in freezer for 10 days and then boiled for 5 minutes in the presence of 1% SDS and 1% mercaptoethanol.
6. Larval extract, boiled for 5 minutes in the presence of 1% SDS and 1% mercaptoethanol soon after homogenization.
7. Pupal extract, incubated at 37°C for one hour with 1% SDS and 1% mercaptoethanol.

FIGURE 6

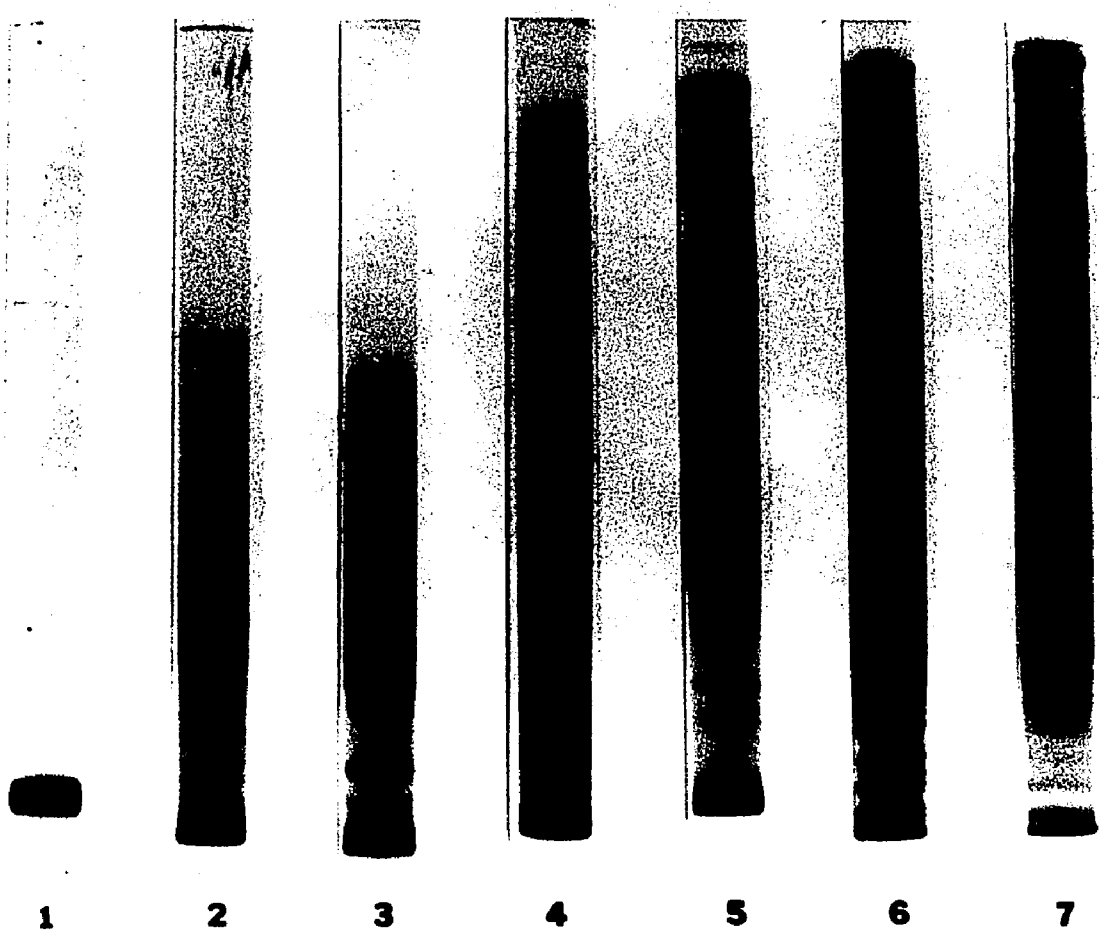


Table 5. Effects of SDS and 2-mercaptoethanol on protein bands of third instar larval homogenate in SDS acrylamide electrophoresis.

Medium added to homogenate	Incubation temperature (degrees centigrade)	Protein bands[1]
Buffer (0.0625 M tris-HCl)	37°[2]	+++
"	25°[2]	+++
"	0°[2]	+++
"	37°, followed by boiling[3]	++++
1% SDS in buffer	37°	-
"	25°	-
"	0°	++
"	37°, followed by boiling	-
1% 2-mercaptoethanol in buffer	37°	+++
"	25°	+++
"	0°	+++
"	37°, followed by boiling	++++
1% SDS + 1% 2-mercaptoethanol in buffer	37°	-
	25°	-
	0°	++
"	37°, followed by boiling	-
"	Boiling for 15 min	++++

[1]++++ = whole gel banded.
+++ = bottom 3/4 banded.
++ = bottom 1/2 banded.
- = no bands.

[2]Incubated for one hour at temperature indicated.

[3]After one-hour incubation at 37°c, sample was boiled for 5 min in the presence of 1% SDS and 1% 2-mercaptoethanol.

buffer or mercaptoethanol is not due to the removal of proteins during incubation. Their absence, however, could be due to the presence of SDS in the electrode buffer and/or in the gel.

Pupal extracts treated in any of the ways of treating larval extracts gave identical banding patterns (Figure 6, 7). It thus appeared that proteolytic activity was present only in larvae under the conditions used. This speculation was confirmed by the experiment of mixing larval and pupal extracts and adding SDS. Following this treatment, gels were devoid of protein bands.

Proteolytic enzyme in larval homogenates

Properties of the proteolytic enzyme apparently present in larval homogenates were investigated to determine whether (1) it is a soluble enzyme, (2) its proteolytic activity is present at any specific stage during development and (3) it is located in a specific tissue. In order to test these parameters, the following assay was devised. The sample to be tested for proteolytic activity was incubated with SDS plus mercaptoethanol at 37°C for one hour prior to electrophoresis. After electrophoresis, the gels were scored for full banding or absence of all bands to indicate the presence or absence of proteolytic activity.

The larval whole homogenate was centrifuged at 37,000 g for 35 min at 0°C and both the supernatant and the resuspended pellet were tested for proteolytic activity.

Neither fraction showed any bands on the gels.

Whole homogenates of freshly laid eggs (± 1 hr), late eggs (19 ± 1 hr), or 1-4-day-old pupae all had full banding patterns. Larval extracts from early and late first, second and third instar stages showed no bands. Adult homogenates yielded banding of the bottom half of gels. Absence of bands in the larval extracts suggests that proteolytic activity is present during feeding stages.

Pupal homogenate by itself exhibited the full banding pattern; but when mixed with larval extract it yielded gels showing complete absence of bands, implying that proteolytic enzymes of the larval extract hydrolyzed the proteins in the pupal extract. In order to determine the localization of these enzymes, it was decided to test extracts of different larval tissues on pupal homogenate. In preparing such extracts, hemolymph was collected after cutting through the body wall of larvae in tris-HCl buffer. Care was taken not to contaminate the hemolymph with other tissue constituents. Salivary glands, adipose tissue (fat body) and digestive tract with Malpighian tubles were dissected out and homogenized separately in buffer. Larval tissue homogenates were then individually mixed with pupal homogenate and the mixtures tested for proteolytic activity. Results are summarized in Table 6.

Only extracts of digestive tract possessing Malpighian tubles resulted in complete absence of pupal bands. Next, larval tissue homogenates were incubated separately at

37°C for one hour in the presence of SDS plus mercaptoethanol. Hemolymph, and extracts of salivary glands and fat body yielded bands on the gels whereas extract of digestive tract produced no bands. After boiling, extract of digestive tract did produce bands on gels. These results indicate that under the conditions tested, the proteolytic factor in the larva is localized in the digestive tract with Malpighian tubules.

In another experiment, the larval digestive tract was dissected out and the remainder of the larva was homogenized and tested for proteolytic activity either by itself or mixed with pupal homogenate. Both types of extract yielded bands on the gels. Localization of the proteolytic factor in the digestive tract suggests that it might be a digestive enzyme.

A number of experiments were conducted to determine chemical properties of the proteolytic enzymes in larval extracts. Soy bean trypsin inhibitor in the final concentration of 5×10^{-6}% added to incubation mixtures did not inhibit the proteolytic activity of larval extracts, nor did 0.001 M EDTA similarly added. Change in pH of the homogenization buffer from 6.8 to 7.8 did not alter proteolytic activity. Digestive tract extract from larvae treated with fluorouracil for up to five days showed proteolytic activity. Treatment with fluorouracil under starvation for as long as eight days or more, however, resulted in the appearance of a few low molecular weight

Table 6. Effects of different larval tissues on the protein bands of pupal extract in SDS acrylamide gel electrophoresis after one hour incubation at 37°C in the presence of 1% SDS and 1% 2-mercaptoethanol.

No.	Sample	Protein bands
1	pupae	++++
2	larvae	--
3	pupae + larvae	--
4	pupae + larval fat body	++++
5	pupae + larval hemolymph	++++
6	pupae + larval salivary glands	++++
7	pupae + larval digestive tract	--
8	larval fat body	++++
9	larval hemolymph	++++
10	larval salivary glands	++++
11	larval digestive tract	--

++++ = all bands present.

-- = no bands.

bands on gels (Figure 6, 2).

Storing larval homogenate in the freezer (-4°C) for more than ten days or freeze-thawing it more than twice resulted in absence of thin bands along the length of the gel and low intensity of staining of other bands, even if the homogenate was boiled with SDS plus mercaptoethanol prior to electrophoresis (Figure 6,5).

To limit loss of protein through proteolytic activity during sample preparation, SDS and mercaptoethanol both at final concentration of 1%, were added to the homogenate and the test tube holding the mixture was immediately lowered into vigorously boiling water in a water bath and kept there for 15 - 20 minutes. The undissolved material was removed from suspension by centrifugation at 3000 rpm for three minutes in a clinical centrifuge and the supernatant was stored in the refrigerator. Just prior to electrophoresis, samples were exposed to the boiling water bath for two minutes and then cooled to room temperature before applying to the gels. It was found that freshly made extract and that which had been in the refrigerator for as long as twenty days showed identical results.

Electrophoresis of proteins in fluorouracil-treated larvae

To determine whether fluorouracil-blocked larvae showed the larval type or the pupal type of electrophoretic pattern of protein, the following experiment was conducted. Early and late third instar larvae, 65 (± 1)

and 79 (± 1) hours old, were continuously treated in 0.3 mg FU and 1.2 mg TdR per ml. Appropriate water controls were set up for each age studied. In both ages studied, the water controls went through normal pupation and adult emergence, whereas FU-treated larvae remained as larvae without undergoing pupariation and subsequent development. Larvae treated with FU, and also water controls were collected and homogenized at the start of the treatment and every 24 hours thereafter. Results are shown in Figure 7.

Based on visual observation and densitometric scanning of the gels, a composite diagrammatic representation of gel containing all the protein bands which were resolved for the developmental stages studied is shown in Figure 8. A total of 54 protein bands were identified. Third instar larvae, 65 - or 79 - hours-old, did not yield bands #34 and #43. Water control larvae approaching pupariation (94-hours old) and from then on did yield band #43, and fluorouracil-treated larvae showed band #43 at comparable ages. Band #34, however, was present only in the extracts of pupae of water controls; it was never seen in FU-blocked larvae. Based on the mobility of the standard proteins in 10% SDS acrylamide gel, treated in the same way as larval or pupal samples, the approximate molecular weight of the pupal specific protein (band #34) was 50,000 daltons (Figure 9).

Figure 7. The effect of fluorouracil and thymidine treatment (0.3 mg FU, 1.2 mg TdR / ml) on the general protein banding pattern of Drosophila in SDS 10% acrylamide gel electrophoresis. In all the following the treatment was started when the larvae were 66 (± 1) hours of age.

1. After 32 hr of FU + TdR treatment
2. After 32 hr in H_2O (control). Larvae have formed puparium by this time. The pupal specific band is indicated by arrow.
3. After 55 hr in FU + TdR
4. After 55 hr in H_2O
5. After 98 hr in FU + TdR
6. After 98 hr in H_2O
7. After 125 hr in FU + TdR
8. After 125 hr in H_2O
9. After 9 days in FU + TdR

Note the similarity in the intensity of different bands in all the gels. Pupal extract of 1 - 4 day old pupae (#S, 2, 4, 6 and 8) do not show any difference in banding pattern.

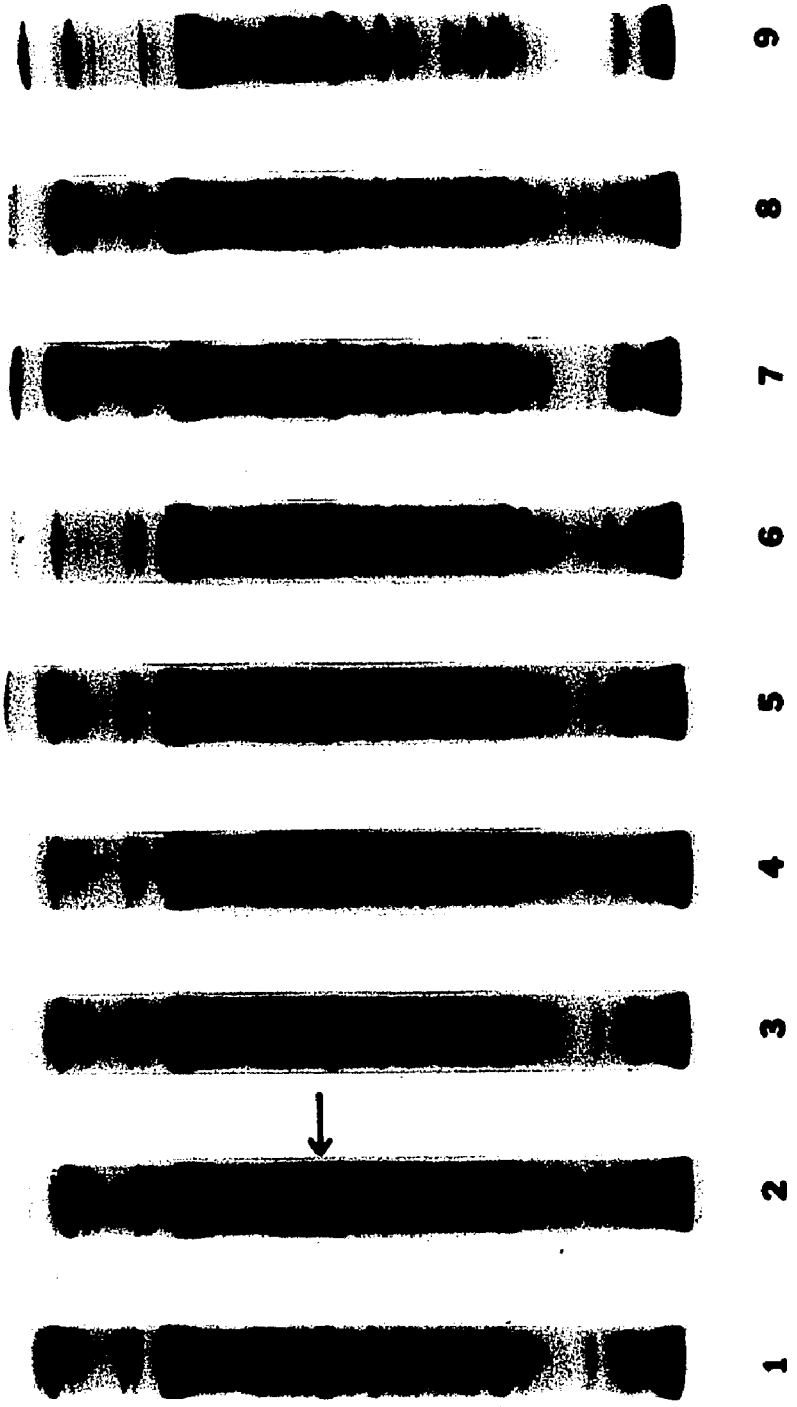


FIGURE 7

FIGURE 8

Schematic representation of gel containing all protein bands encountered during developmental stages of Drosophila, from 65-hour-old larva through 4-day-old pupa.

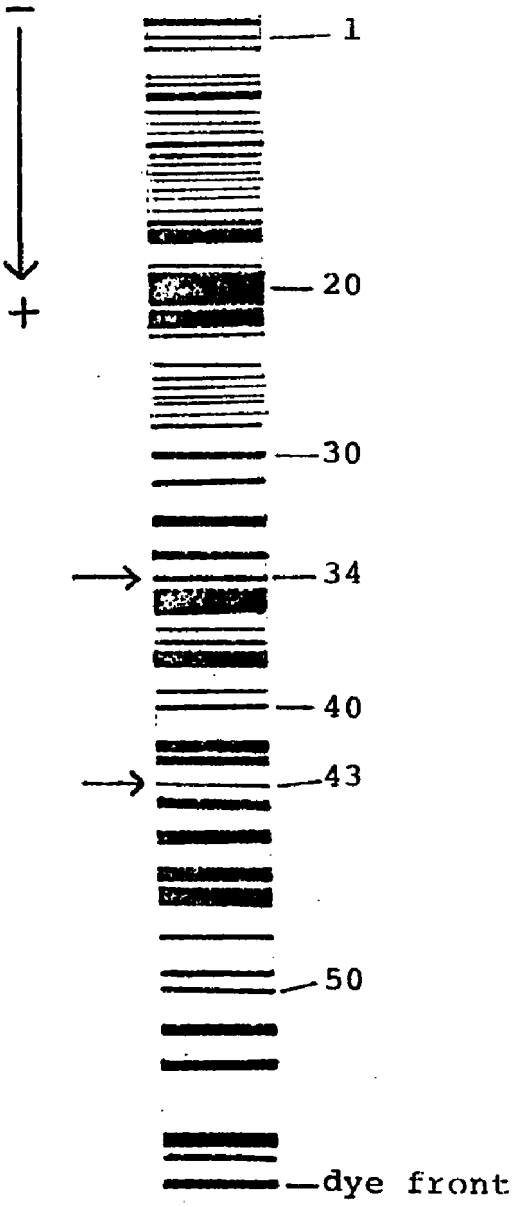


Figure 9. Log of molecular weight versus mobility of five standard proteins of known molecular wt. in SDS 10% acrylamide gel.

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{distance of dye migration}}$$

a. Bovine serum albumin

b. Ovalbumin

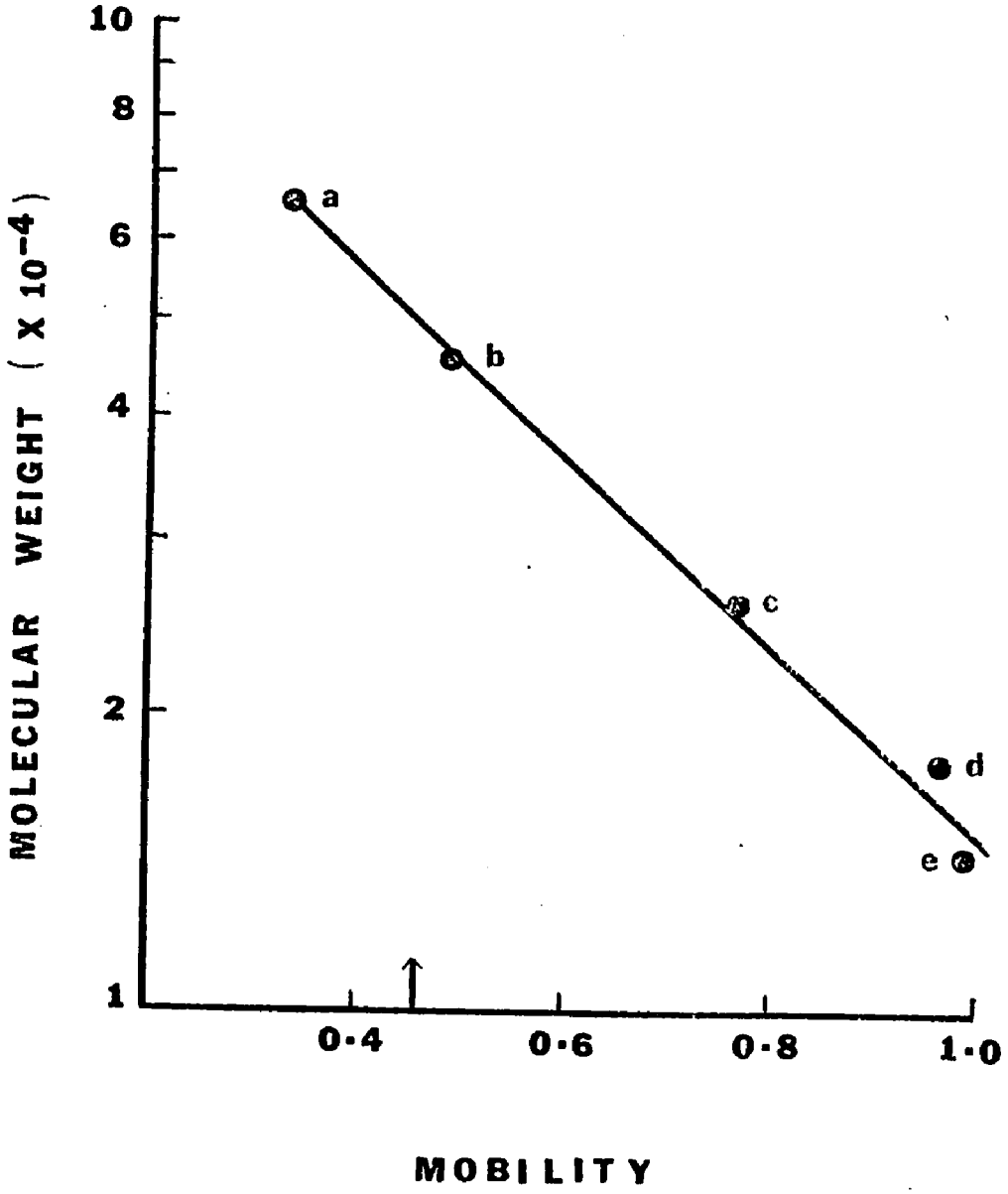
c. Chymotrypsinogen

d. Myoglobin

e. Lysozyme

Arrow indicates the relative mobility of the pupal specific protein.

FIGURE 9



Visual observation and densitometric scanning of gels revealed no obvious quantitative differences between comparable protein bands of third instar larvae and pupae (Figure 7). However, these methods do not preclude the possible existence of minor quantitative differences. Problems in the techniques of measuring protein concentration, of electrophoresis and of scanning, may mask real differences.

Within the limits of sensitivity of the techniques used, a difference of one protein band, #34, distinguishes the late third instar larva and pupa during normal development. One to four-day-old pupae yielded similar protein patterns (Figure 7). Larvae treated with fluorouracil possessed the late larval protein represented by band #43 but never developed band #34, the pupal-specific band.

Isozyme studies

For isozyme studies, third instar larvae, 65 (± 1) and 79 (± 1) hours old, were continuously treated in 0.3 mg FU and 1.2 mg TdR per ml and compared with water controls. Samples were collected at the start of treatment and every 24 hours thereafter. Electrophoresis on 6% acrylamide gels and subsequent staining procedures for different isozymes studied are described in Materials and Methods.

α-esterase

Third instar larval controls from 65 to 90 hours old yielded six isozyme bands (Figure 10, A). Isozymes of

fluorouracil-treated larvae up to 90 hours of age showed the same pattern. Both controls and FU-treated larvae between 90 and 96 hours old (prior to puparium formation in controls) produced isozyme patterns lacking band #4 (Figure 10, B). This pattern persisted in FU-blocked larvae for as long as the larvae survived (10 days after treatment started), but in water controls only up to 20 hr after puparium formation. After this period, in gels of controls, band #2 became broader, band #3 became lighter in staining intensity and band #5 was absent (Figure 10, C). Ten hours later a new isozyme identified by a broader band, #4a, developed in pupae of water controls and it lasted until the adults emerged (Figure 10, D). Newly emerged adults lacked the isozymes of bands #5 and #6, and band #4a had become very light in staining intensity (Figure 10, E). Later the adults no longer had isozyme #4a.

<u>β-esterase</u>

All larvae from 65 hours of age through 94 hours yielded three isozyme bands (Figure 10, F). This pattern persisted for larvae treated with fluorouracil for as long as 9 days after beginning treatment at the age of either 65 or 79 hours. Water controls 20 hours after puparium formation produced a wider, more intense band #2, but a narrower and lighter band #3 (Figure 10, G). Thirty hours after puparium formation, the isozyme for band #4 appeared and it remained until the very late pupal stage.

Figure 10. The effect of fluorouracil and thymidine (0.3 mg FU, 1.2 mg TdR / ml) treatment on isozyme pattern during Drosophila development.

isozymes of α- esterase

A. 65-hour-old larva

B. 94-hour-old larva or FU-blocked larva

C. One-day-old pupa

D. 3-day-old pupa

E. Adults

isozymes of β- esterase

F. 75-hour-old larva, FU-blocked larva show the same pattern

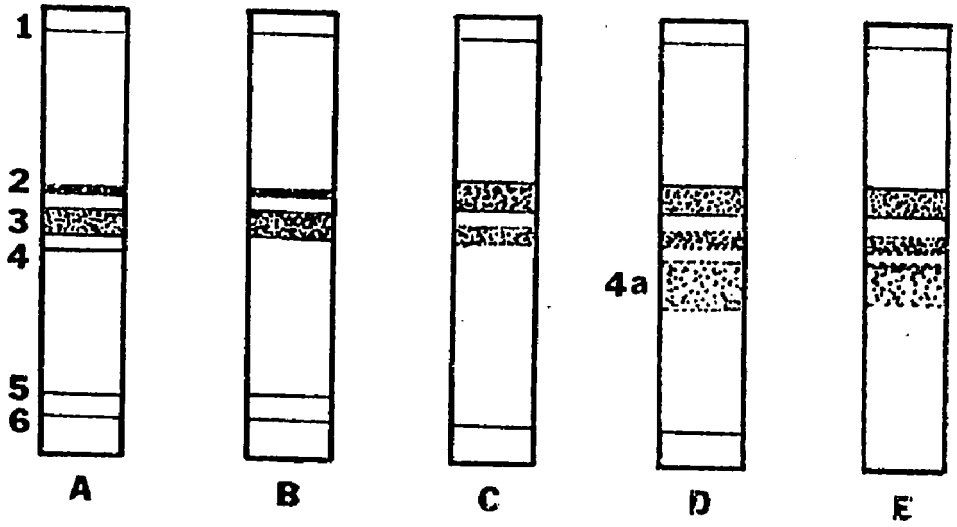
G. 1-day-old pupa

H. 3-day-old pupa

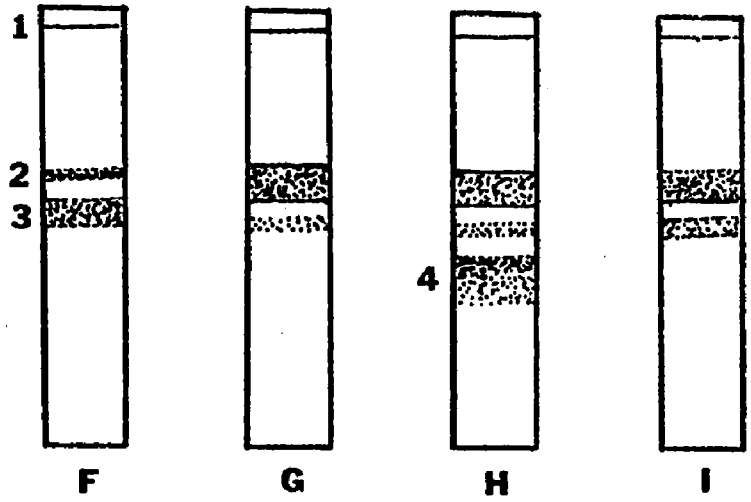
I. Adults

FIGURE 10

α-ESTERASE



β-ESTERASE



(Figure 10, H). Adults lacked the isozyme for band #4 (Figure 10, I).

Alkaline phosphatase

Larvae 65 (± 1) hours old had three isozymes of alkaline phosphatase, yielding bands #1, #2 and #4 (Figure 11, A). Band #4 isozyme had disappeared in larvae after 24 hours in water or treated with fluorouracil (Figure 11, B). Shortly before puparium formation in water controls and at a similar age in FU-treated larvae, the isozyme for band #1 was absent (Figure 11, C). Only the isozyme for band #3 persisted in FU-blocked larvae until they died and the band was narrower and lighter in intensity than for younger larvae (Figure 11, D). In early pupae, a new isozyme producing band #3 appeared (Figure 11, E). Later, pupae had lost the isozyme for band #2 and retained only that for the single band #3 (Figure 11, F).

Acid phosphatase

Early third instar larvae yielded two bands, #1 and #3 (Figure 11, G). After 90 hours of age both water controls and FU-treated larvae lacked the isozyme of band #3. Larvae of the water controls, prior to puparium formation had the isozyme for band #1 only (Figure 11, H); but its staining intensity was darker than that of FU-blocked larvae of similar age. Band #1 persisted in gels of FU-blocked larvae as long as they lived, but it became very narrow (Figure 11, K). Early pupal stages of water

Figure 11. The effect of fluorouracil and thymidine (0.3 mg FU, 1.2 mg TdR / ml) on the isozyme pattern of Drosophila during development.

Isozymes of alkaline phosphatase

A. 65-hour-old larva

B. 90-hour-old larva

C. One-day-old pupa

D. 2 - 4 day-old pupa

E. FU-blocked larva

Isozymes of acid phophatase

F. 65-hour-old larva

G. 90-hour-old larva

H. Larva prior to puparium formation (94-hour-old)

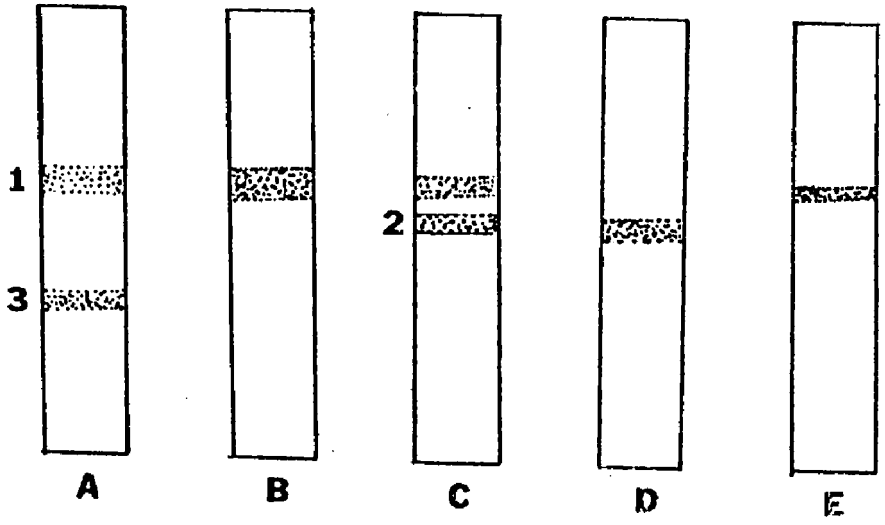
I. FU-blocked larva

J. Early pupa (one-day-old)

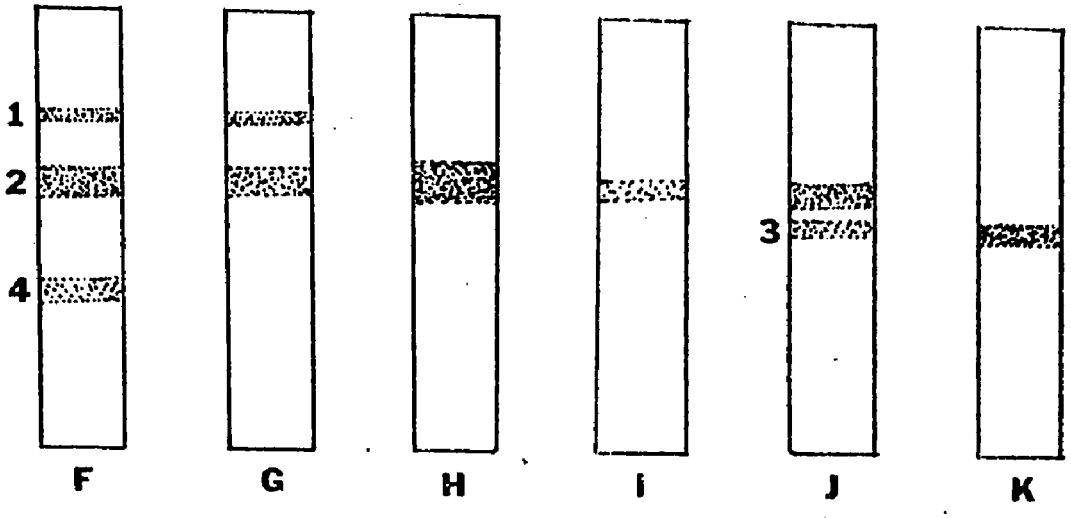
K. Late pupa (3-day-old)

FIGURE 11

ALKALINE PHOSPHATASE



ACID PHOSPHATASE



controls yielded a new band, #2 below band #1 (Figure 11, I). In mid and late pupae, the isozyme for band #1 was absent (Figure 11, J).

Summary of isozyme studies

(1) For each of the four different isozymes studied, there exist three stage specific patterns during the developmental period of interest in Drosophila melanogaster. Early third instar stage exhibits one pattern,which is replaced by the second pattern during late third instar stage and the third pattern emerges during pupal stages.

(2) Water controls went through these stage specific isozyme patterns for all four enzymes studied corresponding to their normal developmental changes in external morphology. Fluorouracil blocked larvae, irrespective of the age at which the treatment started (65 or 79 hours old), showed the first pattern of isozymes and went through the second pattern at a corresponding time when the water control exhibited the second pattern. The third pattern,which was normally seen during the pupal stages of the water control, was never seen in the FU blocked larvae as long as they survived.

(3) Thus, fluorouracil did not block the changes in the isozyme pattern that normally occurred in the larva, but did block the change to the pupal pattern of isozymes, corresponding to its blocking of the larvae from entering into pupariation and subsequent development.

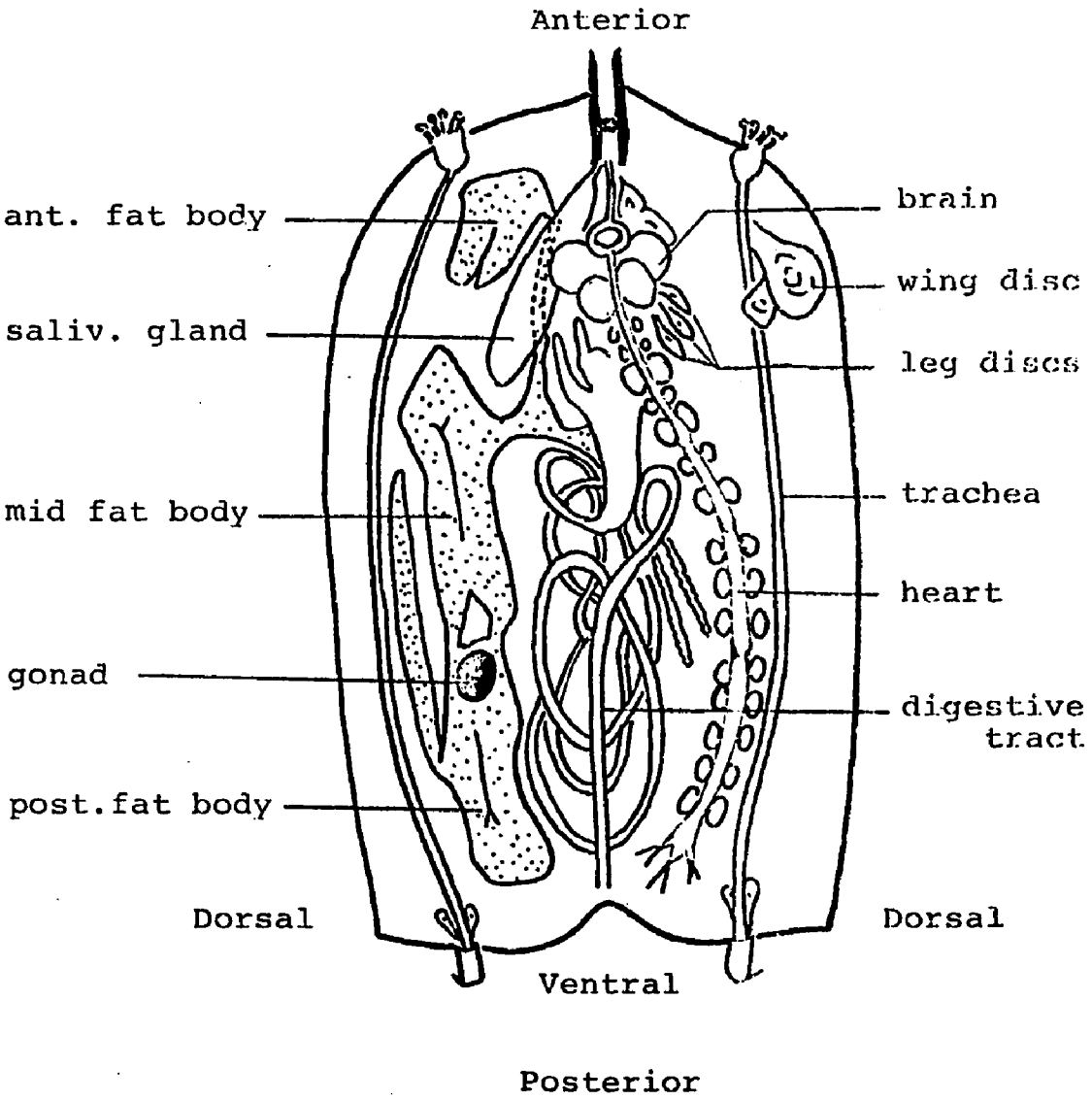
Histochemical studies

At the end of the third instar larval stage (about 96 hours old), the larva of Drosophila begins transformation to the pupal stage during which extensive reorganization of tissues takes place. Histolysis of certain larval tissues and differentiation of adult structures from imaginal discs are obvious changes. Fat body (adipose tissue), salivary glands, testis and imaginal discs were chosen for a histochemical analysis of metamorphic changes in FU-blocked larvae compared with those in controls. Location of these tissues in the larva is shown diagramatically in Figure 12. Their normal developmental changes during metamorphosis, as described for each organ studied in this investigation, are known from previous studies (Bodenstein, 1950).

Early third instar, 66($\pm$ 1) hours old, and late third instar, 80 ($\pm$ 1) hours old, larvae were continuously treated in 0.3 mg FU and 1.2 mg TdR/ml, and controls were cultured in water. Larvae for histochemical analysis were fixed at the start of treatment and at intervals of about 24 hours. Studies were done on control animals up to puparium formation and on FU-blocked larvae living up to ten days after treatment started. For each sampling age, eight larvae were selected randomly from different dishes.

FIGURE 12

A diagrammatic view of the organization of internal larval anatomy (after Rizki and Rizki, 1972)



Fat body (adipose tissue)

The larval fat body is a bilateral multilobed structure consisting of a monolayer of cells limited externally by a basement membrane and extending the length of the larva. During the third instar stage, fat cells increase in volume considerably and accumulate cytoplasmic deposits of lipid, glycogen and protein. Lipid and glycogen accumulate throughout all the larval instars, whereas proteinaceous globules do not start to develop until midway through the third instar stage. Protein globules increase in size and number until just prior to pupation (Butterworth et al., 1965). Shortly after pupation the basement membrane dissolves, and cells of the fat body separate from one another. Few of them histolyze during the pupal stage, but many persist into the adult stage (Bodenstein, 1950). Adult adipose tissue arises during metamorphosis from cells contained in hypodermal imaginal discs, and the adult fat cells contain deposits of glycogen and lipids but no proteinaceous globules (Bodenstein, 1950; Butterworth et al., 1965).

In the histochemical studies of larval fat body, attention was given to the development of protein globules in fat cells. In 66-hour-old larvae, anterior, middle and posterior regions of the fat body (see Figure 12) had fat vacuoles and no protein globules (Figure 14B).

When FU + TdR treatment was started at this age and continued for up to 9 days, fat cells did not develop protein globules. These cells gradually shrank in size until the adipose tissue became only strings of small cells (Figure 14C). In water controls established at the age of 66 hours, however, protein globules of variable size and intensity of staining were seen after 24 hours (prior to puparium formation). The protein globules were more abundant and attained larger size in anterior and middle regions of the fat body than in the posterior region.

In 80-hour-old larvae, the fat cells had numerous fat vacuoles and few, very small protein globules distributed throughout the anterior, middle and posterior regions of the fat body (Figure 13A). If FU + TdR treatment was started at this age, larvae treated for 16 hours showed an increase in size and number of protein globules. A similar pattern was also seen in the water control after 16 hours, just prior to puparium formation (Figure 13B). Anterior and middle regions had fat cells with larger protein globules than those in the posterior cells. In larvae treated in FU + TdR for 44 hours, the protein globules in both anterior and middle regions had increased further in size, whereas those in the posterior region remained the same size as at 16 hours (Figure 14A, E, F). This pattern of restricted growth of protein globules posteriorly was maintained during the rest of the

treatment time. In larvae treated for long periods (6 days to 11 days) the fat cells diminished in size, and fat vacuoles disappeared. Cells in the anterior and middle regions of the fat body had indistinguishable cell boundaries and were filled with protein globules (Figures 14D, 13C, D, E, F).

Figure 13. The effect of FU + TdR on the development of proteinaceous globules in the fat body of Drosophila larva.

A. Mid fat body 80 (± 1)-hour-old larva. Note the small dark granules distributed in the cytoplasm of the cells. Nuclei stain dark blue. x 600

B. Mid fat body of a larva treated in H_2O (control) for 16 hours, starting at 80 (± 1) hours old. Protein globules are of variable size and staining intensity. x 600

C. Mid fat body region of larvae treated with FU + TdR for 65 hours, starting at 80 (±1) hours old. x 158

D. Higher magnification of a portion of Figure 13C. x 600

E. Mid fat body of a larva, treated in FU + TdR for 5 days, starting at 80 (± 1) hours old. x 158

F. Higher magnification of a portion of Figure 13E. x 600. Compare with Figures 13A, B and D for the degree of development of proteinaceous globules and reduction of fat vacuoles.

Bar in Section E represents 50 u.

Bar in Section F represents 10 u.

FIGURE 13



Figure 14. The effect of FU + TdR on the fat body and wing imaginal disc of Drosophila larva.

A. Section of mid and posterior regions of fat body of FU-blocked larva. Treatment with FU + TdR started when larva was 80 (± 1) hours old and continued for 44 hours. Mid fat body (upper half of the picture) shows larger and more protein globules whereas posterior regions of fat body (lower half of the picture) contains fewer and smaller protein globules. The empty circles represent fat vacuoles. x 158

B. Mid fat body of 66-hour-old larva. Size of cells is indicated by distance between darkly stained nuclei. x 158.

C. Mid fat body of a larva treated in FU + TdR for 7 days, starting at 66 (± 1) hours old. Cells are much smaller than those shown in Figure 14B. x 158

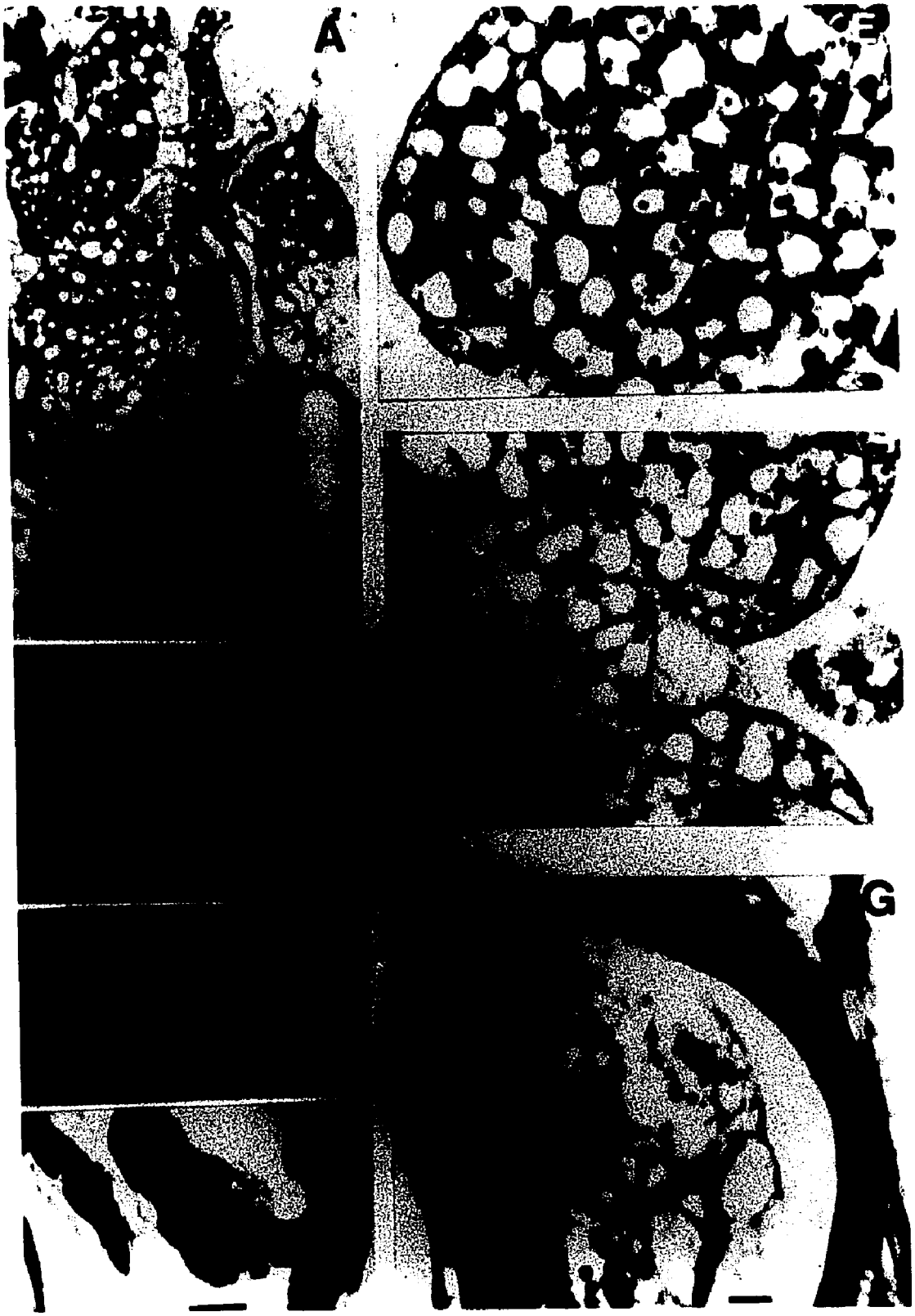
D. Mid fat body of a larva treated in FU + TdR for 10 days, starting at 80 (± 1) hours old. Cells are filled with dark proteinaceous globules, but contain no fat vacuoles. Cell boundaries are indistinguishable. x 158. Bar represents 50 u.

E. Higher magnification of mid region of fat body shown in Figure 14A. x 600

F. Higher magnification of posterior region of fat body shown in Figure 14A. x 600

G. Portion of a wing disc from a larva treated in FU + TdR for 7 days, starting at 80 (± 1) hours old. Note the pycnotic nuclei, staining intensely blue along the cell layer and inside the lumen. x 600 Bar represents 10 u.

FIGURE 14



Salivary glands

The major function of the larval salivary glands of Drosophila melanogaster is the production of "puparial glue", which attaches the puparium to the substratum. Ultrastructural investigations (Lane et al., 1972 ; Gaudecker, 1972) have shown that the secretion begins to form about the middle of the third instar stage and is extruded into the gland lumen 3 hours before puparium formation. Larval salivary glands undergo histolysis during the pupal stage, and by 24 hours after puparium formation the larval salivary gland is dissolved. The adult salivary glands develop from the ring of imaginal cells present at the proximal end of the larval salivary gland (Bodenstein, 1950).

Treatment started at 66 hours

In 66-hour-old larvae the salivary gland cells showed uniform intensity of staining in the cytoplasm. Twenty-four hours later in the water control, these cells had increased in size and their cytoplasm contained distinctly stained protein granules. In FU-treated larva there was only a slight increase in size of the salivary gland cells and they did not develop distinct protein granules. Cytoplasm appeared vacuolated after 60 hours of FU treatment, but the salivary glands persisted throughout FU treatment.

Treatment started at 80 hours

Both water controls and FU-treated larvae showed a similar increase in the size of cells and deposition of protein granules after 16 hours of treatment (Figure 15A, E). In FU-treated larvae, 44 hours after the treatment started the cytoplasm appeared vacuolated and the gland lumen contained a bluish mass (Figure 15C, F). Forty hours later the nuclei appeared pycnotic and large vacuoles had developed in the cells (Figure 15D, G). After 9 days of treatment in FU+TdR, the distal cells had become degenerate (Figure 15H).

Figure 15. The effect of FU + TdR treatment on the salivary glands of Drosophila larvae, starting at 80 (± 1) hours old.

A. Salivary gland (left half of the picture) of a larva treated in H_2O for 16 hours prior to puparium formation. Cells are filled with numerous small protein granules. x 600. Bar represents 10 u.

B. Salivary gland of a larva, treated in FU + TdR for 30 hours. x 600

C. Salivary gland of a larva, treated in FU + TdR for 65 hours. Note the vacuolated appearance of the cells. x 600

D. Salivary gland of a larva treated in FU + TdR for 6 days. Note darkly stained pycnotic nuclei. x 600

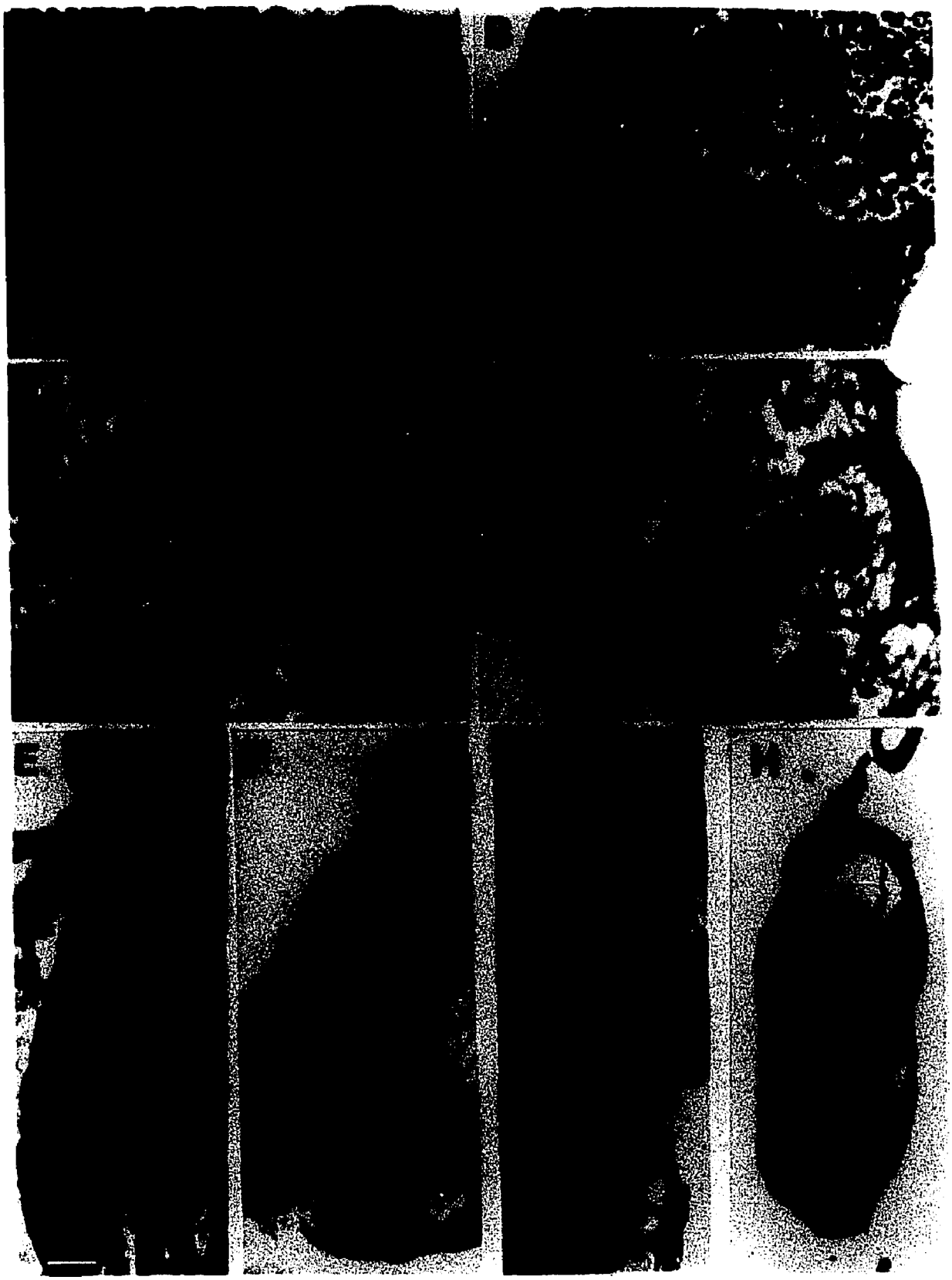
E. Lower magnification of section shown in Figure 15A. x 158. Bar represents 50 u.

F. Lower magnification of section shown in Figure 15C. Note the darkly stained substance, presumably puparial glue, in the gland lumen. x 158

G. Larva treated in FU + TdR for 8 days. Note large vacuoles developing in salivary gland cells. x 158

H. Salivary gland of a larva treated in FU + TdR for 11 days. Degenerate distal cells stain as irregular dark masses. x 158

FIGURE 15



Testis

In the testis of late third instar larvae of Drosophila melanogaster, spermatogonia are localized at the anterior tip, while spermatocytes fill the rest of the organ. Twenty-four hours after puparium formation, nearly mature spermatozoa are present, in addition to earlier stages of spermatogenesis. The testis elongates and assumes a spiral shape after it becomes attached to the vas deferens, which is developed from a genital disc. At emergence of the fly, the testis contains mostly spermatozoa and only few cells in the earlier stages of spermatogenesis (Bodenstein, 1950).

Early and late third instar larvae have testes which show conspicuous intercellular spaces among the cells (Figure 16A). In late third instar larvae (prior to puparium formation in control larvae or at a corresponding stage in FU+TdR treated ones irrespective of the age at which treatment was started, cells staining dark blue and surrounded by empty space, presumably degenerate spermatogonia (Cooper, 1950), were seen (Figure 16B).

The size of the testis remained the same in FU+TdR-treated larvae from the start of treatment until mortality. After 5 days of continuous treatment, whether treatment was started at 66 or 80 hours of age, sperm bundles started to appear (Figure 16C,D). After 6 days of

Figure 16. The effect of FU + TdR on the differentiation of testis in Drosophila larva.

A. Testis of a larva after 16 hours of FU + TdR treatment, starting at the age of 80 (± 1) hours. Note the cellular nature of the testis. x 158

B. Higher magnification of section similar to that in Figure 16A. x 600

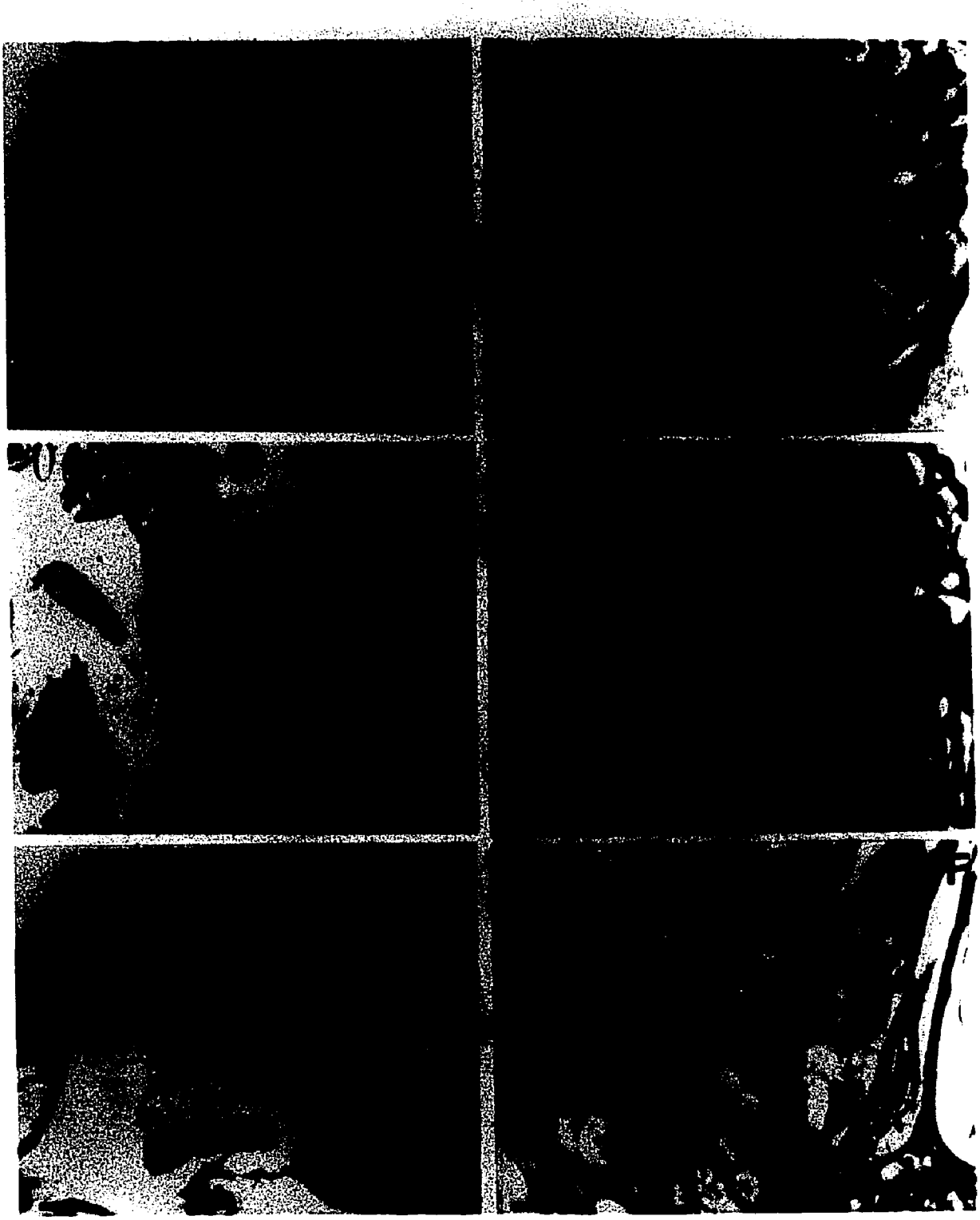
C. Testis of a larva after 65 hours of FU + TdR treatment, starting at 80 (± 1) hours of age. x 158

D. Higher magnification of a portion of Figure 16C. Some of the cells in the lower right corner have become fibrillar and may be spermatozoa. x 600

E. Testis of a larva treated in FU + TdR for 6 days, starting at 66 (± 1) hours old. The cellular nature of the testis (Figure 16A) is not seen here. x 158. Bar represents 50 u.

F. Testis of a larva treated for 6 days in FU + TdR, starting at 80 (± 1) hours old. The testis is filled with bundles of fibrillar structure, presumably spermatozoa. x 600. Bar represents 10 u.

FIGURE 16



treatment, the whole testis contained bundles of fibrillar structures, presumably sperm bundles (Figure 16E, F).

Imaginal discs

No change in size or shape was noticed in imaginal discs if FU+TdR treatment was started at 66 (± 1) days during an early third instar stage. When treatment was started later (80± 1 days), a slight increase in size of wing and leg discs was observed; also the wing disc seemed to unfold into a small balloon-like structure. These changes were observed in sections as well as in dissections. Since the significance of this unfolding is not known, it will not be discussed further here. After about 6 days of treatment, (starting at either 66 or 80 hours), the disc cells appeared pycnotic and cell layers seemed to be discontinuous, some cells becoming free inside the lumen (Figure 14G).

Summary of histochemical studies

1. Fluorouracil treatment elicited different responses in the fat body of third instar larvae of Drosophila, depending upon the age at which treatment started. When FU treatment was started at an early third instar stage (66-hour-old larva), the fat body did not develop protein globules. When treatment was started in the late third instar stage (80-hour-old larva), however, protein globules did develop in the fat body and the globules increased in size. The protein globules persisted as long as the larva survived in FU treatment. The fat body exposed to fluorouracil, starting either at 66 or 80 hours, never underwent the histolysis characteristic of pupal stages during normal development. Fat cells shrank in size after long treatment (10 days), possibly because the reserve material in fat cells was needed for survival of the larva.

2. Salivary glands responded similarly to the fat body during FU treatment. Treatment begun early (66 hours old) prevented the normal increase in size of cells and the development of protein granules, whereas treatment started later (80 hours old) did not inhibit growth of cells and their protein globules, nor did it prevent secretion of globules into the lumen. Salivary glands did not undergo histolysis in FU-treated larvae, irrespective of the age at which the treatment started.

However, the developmental changes that normally occur during the pupal stage, for instance, histolysis of these tissues, was blocked by FU.

3. Fluorouracil had no effect on development of the testis. Whether FU treatment was started early or late, larvae developed the sperm bundles which normally form during the pupal stage.

4. Imaginal discs did not differentiate in FU-blocked larvae.

DISCUSSION

It has been shown that major ontogenetic events in insect development, namely growth, molting, pupation and adult development are under both genic and hormonal control (Wigglesworth, 1954; Wyatt, 1972; Doane, 1972). Drosophila development has been reviewed by a number of workers (e.g., Poulson, 1950; Sonnenblick, 1950; Bodenstein, 1950; Ashburner, 1972; Berendes, 1965; Mitchell, 1967; Fristrom, 1970; Dickinson, 1975); however, the genic and humoral controls during metamorphosis have not been fully elucidated.

Lethal mutants of Drosophila are useful in the classical approach to study genes in development (Hadorn, 1955). Stages of visible deviation between mutant and normal in the course of development are described as effective lethal phase. However, long before these phenotypic differences in visible pattern in a mutant set in, biochemical differences arise and such differences can be shown by using physical or chemical stimuli to interfere with the expression of mutant pattern or by inducing phenocopies in wild-type individuals. The period of development during which such stimuli become effective is called the phenocritical period.

With the advent of modern techniques, it is becoming apparent that the primary gene action involves mRNA

synthesis, which in turn is responsible for protein synthesis. Hence, changes in the protein pattern during development are reflective of changing patterns in gene activity. These changes must be taking place during phenocritical periods of development. In the present study, 5-fluorouracil has been used as a probe for investigating certain morphological and biochemical changes during metamorphosis of Drosophila melanogaster. Five-fluorouracil is an analog of uracil and can be incorporated into RNA (tRNA, rRNA and mRNA). The use of this analog under controlled conditions can give important information as to how gene action is related to the variety of steps involved in development and differentiation.

Effect of fluorouracil treatment

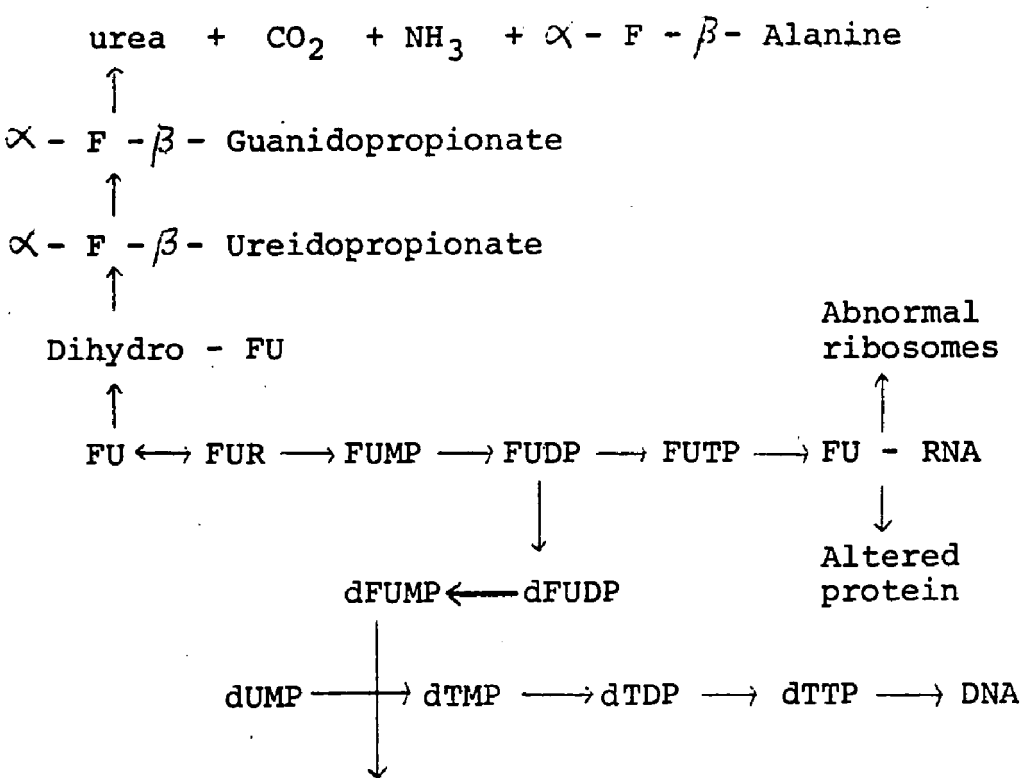
In mammalian and bacterial systems, 5-fluorouracil (5-FU, FU) is converted to its ribonucleoside, fluorouridine (FUR) by uridine phosphorylase. In a series of phosphorylation steps, FUR is further changed to FUMP $\longrightarrow$ FUDP $\longrightarrow$ FUTP. Formation of FUMP may also result from direct conversion of FU to FUMP by uracil phosphoribosyltransferase. FUTP is incorporated into RNA and this fraudulent base-containing-RNA may cause aberrations in enzymes, proteins, or in structural organization of ribosomes (Gros and Naono, 1961; Champe and Benzer, 1962; Aronson, 1961). FUDP is also converted to its deoxyribonucleotide, dFUMP which is a specific and potential inhibitor of thymidylate

synthetase. The inhibitor blocks the de novo synthesis of TTP from dUMP and consequently inhibits DNA replication (Cohen et al., 1958; Heidelberger et al., 1957; review by Heidelberger, 1965; Burman, 1970; Mandel, 1969). A scheme of FU metabolism and sites of inhibition is shown in Figure 17 (from Burman, 1970). However, exogenous TdR is available and TdR is phosphorylated to TTP which unblocks DNA replication. These metabolic steps described are known to be present in Drosophila, as demonstrated in a series of experiments (Rizki et al., 1972, 1973, 1975; Carpenter, 1973, 1974; Clynes and Duke, 1975).

In bacterial systems, it has been shown that the inhibitory effects of FU on DNA synthesis can be reversed by the addition of exogenous thymidine in equimolar concentration (Aronson, 1961). In sand dollar embryos, FU blocks the development at an early blastula stage and this effect is prevented by exogenous thymidine or thymine (Karnofsky and Basch, 1959). In Drosophila larva, in the presence of FU, an enhanced incorporation of exogenously supplied thymidine into DNA has been demonstrated (Carpenter, 1973). A study of specific activity of thymidylate synthetase as a function of age in Drosophila development indicated that it is low during the third instar larval stage and prior to puparium formation (Carpenter, 1973). In the present study, simultaneous addition of exogenous thymidine at a concentration twofold that of FU, did not

FIGURE 17

Metabolism of FU and its sites of inhibition



reverse the adverse effects of FU on Drosophila development. Hence, the lethal effects of FU on Drosophila development may not be due to its inhibitory effect on DNA synthesis.

The present results also indicate that co-administration of uridine (UR) or UR plus thymidine (TdR) along with FU does not reverse the developmental arrest in Drosophila by FU. In microorganisms, it has been shown that administration of uracil (U) or UR together with FU prevents the alteration of RNA synthesis produced by the analog alone and also reduces incorporation of FU into RNA (Mandel, 1969). The combination of U with TdR or thymine (T) overcame all the inhibitory actions of FU in microbial systems (Reich and Mandel, 1966; Cohen et al., 1958). The failure of UR and TdR to reverse the effect of FU in the present study may be due to the following: (1) uridine phosphorylase in Drosophila larva has higher affinity for fluorouridine (FUR) than for uridine (UR). (2) Phosphoribosyltransferase has very high activity in converting FU to FUMP. In either case, it will increase the amount of FUTP compared to UTP in the nucleotide pool, leading to the enhanced incorporation of fraudulent base into RNA. (3) The metabolic products of FU, namely α- F -β -ureidopropionate or α- F -β alanine may be responsible for some effects (Jenkins and Simmons, 1968). (4) FU may be more easily penetrated and/or more stable than U. In human cells growing in tissue culture, neither TdR nor UR in different concentrations, prevented

the inhibitory effects of FU (Rich et al., 1958).

The results reported herein do not indicate the specific level at which FU is exerting its effects on Drosophila development. It is difficult to compare the effects of FU and reversal studies in microbial systems with those in multicellular systems like that of a Drosophila larva. Since ^{3}H - labelled FU has been shown to be incorporated into RNA of Drosophila larva (Rizki, 1968), it is possible that FU does have an effect at the RNA level.

The results of FU treatment starting at different ages during the third instar stage may indicate that the FU-sensitive critical phase for producing permanent larvae may be over by about 85 hours of age. Normally larvae cease to feed around 85 - 90 hours of age and become quiescent. Hence, the short period of about 5 hours of ingestion of FU if treatments began at 85 hours does not result in an effective threshold concentration for exerting an adverse effect on ontogenetic processes, viz., larval pupal transformation. In experiments on concentration effects, a concentration of 150 ug/ml of FU causes the production of 93% permanent larvae, while a concentration of 18.8 ug/ml of FU administered to larvae of the same age results in no permanent larvae. Hence concentration of fluorouracil and time of its action are undoubtedly important variables affecting its physiological action.

Histochemical studies

Effect of FU on larval adipose tissue

During the development of Drosophila larva, two important processes take place. Cells of adipose tissue and salivary glands show increased activity of protein synthesis and these cells grow in size. Imaginal discs grow by cell multiplication.

The insect fat body (adipose tissue) is a site of storage for lipids, carbohydrates and proteins as well as a site of intermediary metabolism, and is often compared to mammalian liver in function (Kilby, 1963, 1965; Chen, 1966, Price, 1973). In Drosophila, larval fat body develops from a portion of somatic mesoderm of the embryo (Poulson, 1950). The number of cells in the larval adipose tissue remains constant from the time of hatching, but the size of the cells increases throughout larval development (Bodenstein, 1950). The larval fat body is a bilaterally symmetrical, multilobed, monolayer of cells extending along the length of the larva and enclosed by basement membrane. Exposure of the surface of this tissue to the surrounding hemolymph facilitates exchange of metabolites.

During the pupal stage, the basement membrane breaks down, fat body cells become free and some of them undergo histolysis. Presumably the products of histolysis are utilized by the differentiating imaginal tissues (Bodenstein, 1950). The non-histolyzed cells survive through the pupal stage and first two days of adult life. By the

fourth day of adult life, no larval cells remain. The adult adipose tissue arises during metamorphosis from the cells contained in the hypodermal imaginal discs. Larval adipose tissue contains deposits of glycogen, lipids, and proteinaceous globules while adult adipose tissue has deposits of only glycogen and lipids.

In a detailed light and electron microscope study on Drosophila larval fat body, Gaudecker (1963) has shown that the proteinaceous reserves which begin to accumulate in the fat body during mid third instar stage, are autophagic vacuoles containing mitochondria, glycogen and other cellular components enclosed in common membrane. Throughout larval development in Drosophila, lipid and glycogen are accumulated in the fat body; but the proteinaceous globules do not begin to form until midway through third instar stage (24 hours before puparium formation according to Butterworth et al., 1965; 17 ± 2 hours before puparium formation according to Thomasson and Mitchell, 1972). These globules increase in size and number until they comprise about 25% of the cytoplasm prior to puparium formation, and they vary in size, shape and ability to bind protein stain (Butterworth, et al., 1965).

The accumulation of fat body protein reserves in the larval instar of endopterygote insects has been observed by several investigators (review by Price, 1973). The nature and origin of these protein reserves is a subject of controversy. According to Locke and Collins (1965),

protein in the globules in the fat body of the butterfly, Calpodes ethilus, is sequestered from hemolymph. According to Ichizaki (1965) and Walker (1966), in the moth, Philosomia, as in Drosophila (Gaudecker, 1963), the proteinaceous globules are formed from fat body mitochondria and other organelles by action of lysosomes. Following the report of Locke and Collins (1965) in Calpodes, Thomasson and Mitchell (1972) have demonstrated by injecting ^{3}H - labelled proteins and ^{14}C - labelled amino acids that the developing protein globules in the third instar larva of Drosophila incorporated protein but not free amino acids, indicating that the proteins in globules are sequestered from the hemolymph and not synthesized in the fat body cells at the time of granule formation.

In the present investigation, FU treatment started at 66 hours was successful in blocking the formation of protein globules in the fat body even if the larvae remained alive for 10 days. In the control group, the larval fat cells developed globules that increased in size until puparium formation. However, when FU treatment was started at the age of 80 hours, the fat body cells at the beginning of treatment had very tiny protein globules which were sparsely distributed in the cytoplasm. During subsequent continuation of FU treatment, the globules increased in size, comparably to those in water controls at the time of puparium formation. By the end of the seventh to eighth day of FU

treatment, the density of protein globules increased. This was mainly due to the loss of lipid globules and overall reduction of cell volume.

That the formation of protein globules in the larval fat body cells of Drosophila is under hormonal control, most likely by ecdysone, has been shown in a series of transplantation experiments by Butterworth, et al., (1965, 1967). According to these authors, when the fat body of a 48-hour old larva (late second instar) is transplanted into an adult male, protein globules do not develop but when larval ring glands are added as a source of ecdysone, the fat cells develop protein globules. The success of protein globule formation depends upon the competence of adipose cells, which is reached by 48 hours. In another series of experiments, Thomasson and Mitchell (1972) have also shown that injection of α-ecdysone into early third instar larva induced precocious appearance of protein globules. Furthermore, induction of protein globule formation in the fat body of early third instar larvae in vitro in Schneider's medium by addition of α-ecdysone has been demonstrated (Thomasson and Mitchell, 1972); but there is a lag period of 6 hours between the addition of the hormone and the beginning of protein globule formation.

The present investigation shows that FU treatment starting at 66 hours of age blocks the formation of protein globules. This result is likely due to the effect of FU on hormonal secretory activity of the ring gland. These results can

further be analysed in view of the report by Thomasson et al. (1972) who demonstrated that there is a 6 hour lag period between addition of ecdysone and protein globule formation. If this lag period also exists in the mid third instar larva between the ecdysone stimulus and protein globule formation, then our results indicate that an 80-hour-old larva has passed that critical stage, since starting FU treatment at the age of 80 hours, did not prevent the formation of protein globules. It is interesting to note that starting FU treatment at 80 hours of age blocked the puparium formation and subsequent development; yet it failed to prevent protein globule formation in the adipose cells. If pupation is dependent upon a certain titer of ecdysone, then the maintenance of the same titer is not necessary for the continued development of protein globules. It may be that a sustained stimulus by ecdysone is unnecessary for development of protein globules once they appear in the cytoplasm.

The difference in the degree of development of proteinaceous globules in the posterior region as compared to the other areas of the fat body in FU-treated larvae may imply that the cells of this tissue are not homogeneous. Bodenstein (1943) has demonstrated that different imaginal discs have different levels of competence at a given time in development, to react to metamorphosing hormone. The functional heterogeneity between different regions of the larval fat body of Drosophila has been shown in the study

of kynurenine autofluorescence globules (protein) in the different regions of the fat body and in different mutants (Rizki, 1969, 1972). In addition, the appearance of proteinaceous globules in the anterior fat body precedes that of posterior fat body (Rizki, 1969). Systematic differences in the formation of protein globules in the anterior and posterior fat cells among FU-treated larva in the present study reflect an interference with the development of competence to react to hormonal stimuli.

During metamorphosis of Drosophila, the larval fat cells undergo histolysis. Transplantation experiments (Butterworth et al., 1967, 1973; Butterworth, 1972) indicate that histolysis of larval fat cells is coupled with protein deposition. Histolysis occurs only when protein globules are formed, and the extent of histolysis seems directly proportional to the amount of protein. Fluorouracil treatment starting at 66 hours of age prevented the formation of protein globules as well as histolysis. Starting FU treatment at the age of 80 hours, however, permitted the formation of protein globules and yet no histolysis of cells occurred. Thus, FU treatment has uncoupled the two processes, namely, protein globule formation and cytolysis of adipose cells. Therefore, protein globule formation per se may not be responsible for cytolysis. There must exist another RNA dependent triggering mechanism for cytolysis (perhaps cytolytic enzymes). Whether it is the lysosomal enzyme(s) or extra lysosomal lytic

enzyme(s) is not known. Normally, the protein globules are considered to be autophagic bodies bound by membranes (Gaudecker, 1963) and have been shown by histochemical studies to contain acid phosphatase (Butterworth, 1972). However, according to Yagi (1962) in Drosophila, the loss of protein globules from the adipose cells is not due to lysis, but due to extrusion of these globules into the extracellular space.

Lytic ability of fat cells possessing protein globules is a function of the environment and not the property of these cells (Butterworth, 1972). This was demonstrated by implanting fat cells of the mutant ap^4/ap^4, which contain protein reserves but do not lyse during metamorphosis in the mutant environment, into a wild type host where they lyse readily. It appears that prolonging the larval type of environment under the experimental condition of FU treatment is inhibitory to the mechanism which elicits a lysis response in fat cells.

Salivary glands

Fluorouracil treatment, started at the age of 66 hours, inhibited the growth in size of salivary gland cells and also resulted in the development of fewer stored protein granules. Starting FU treatment at 80 hours of age, however, did not diminish the size of cells nor the development of large numbers of protein granules, compared with those in water controls. At comparable ages prior to puparium formation, salivary gland cells in both water

controls and larvae treated with FU beginning at 80 hours secreted proteins, presumably puparial glue proteins, into the gland lumen. These proteins persisted in the lumen as long as the larvae survived. On the other hand, the salivary gland lumen in larvae treated with FU beginning at 66 hours contained no protein at any time during continuation of treatment.

The above results indicate that the information (messenger RNA) necessary for initiating glue protein synthesis was already present in larvae of 80 hours of age but that in 66-hour-old larvae, the RNA coding for glue probably had not yet been synthesized. Starting FU treatment at the earlier age evidently inhibited transcription and thus the synthesis of these proteins. Electrophoretic investigations indicate that saliva begins to be formed about the middle of the third instar stage at around 72 hours of age (Korge, 1976).

Besides the synthesis and secretion of glue proteins, the salivary gland of Drosophila is suspected to have some other functions after puparium formation (Fraenkel and Brooks, 1953; Rizki, 1967; Lane et al., 1972; Gaudecker and Schmale, 1972). Analysis of DNA, RNA and protein composition of this tissue is consistent with the supposition that the gland continues to function until histolysis predominates, about 12 hours after puparium formation (Boyd and Logan, 1972). Changes in the chromosomal puffing pattern in the salivary gland during the prepupal stage support this view

(Ashburner, 1972; Korge, 1975). Geiger and Mitchell (1966) described a component of polyphenol oxidase being synthesized in the salivary glands of the prepupa. Finally, prior to autolysis of the cells in the prepupal stage, large vacuoles develop in the salivary glands, a result interpreted to be a sign of beginning autolysis of salivary glands (Bodenstein, 1950; Lockshin, 1969). The lysosomal nature of these vacuoles was suggested by Lockshin (1969). The salivary glands of FU-treated larvae, after the onset of treatment at 66 or 80 hours of age, proceed to the stage of vacuole formation but never undergo autolysis even when the larvae have passed the physical age of adult (as compared to control group). Here again, as in adipose cells, FU has blocked some triggering mechanism which eventually results in autolysis of cells. The nature of the trigger has been elucidated in Bodenstein's (1943) transplantation experiments. On the basis of these experiments, Bodenstein (1943) concluded that the histolysis of larval salivary gland in Drosophila melanogaster is under the control of ring gland hormone, most likely ecdysone. Glands must reach a state of competence to respond to the hormone and normally, the competence is reached by early third instar stage. Hence, the failure of histolysis in the FU-treated larva may indicate either (1) that the hormone level was prevented from reaching an effective level for the normal histolysis of this tissue, or (2) that the activation of the lytic factor is somehow prevented

from developing by FU.

Testis

It is interesting that in an environment deleterious for normal metamorphic events such as growth and differentiation of imaginal discs and ovaries, or for breakdown of larval tissues such as fat body and salivary glands, the differentiation of spermatocytes into sperm bundles was not prevented. In FU-treated larvae, whether the treatment was started at the age of 66 hours or 80 hours, sperm bundles started to appear at about 120 hours of development. As development proceeded, spermatocytes began to develop into sperms. Shape and size of the testis did not change from the time treatment started until the death of the larvae. In the water controls no sperm bundles were noticed up to puparium formation.

Under normal conditions the testis of third instar larvae contains mainly spermatogonia and primary spermatocytes (Cooper, 1950; Bodenstein, 1950). Just prior to puparium formation, meiosis becomes active and fully formed non-motile spermatozoa are present 24 hours before adult eclosion. Mature sperms have been noticed in mature larvae in some instances (Huettner, 1930). The testis is fully formed at the time of eclosion, and contains germ cells in all stages of spermatogenesis, including sperms (Bodenstein, 1950).

During pupal stages in Drosophila melanogaster, the genital disc located posterior to the gonads differentiates

into vas deferens, and when this duct comes in contact with the developing testis it induces the testis to elongate and assume a spiral shape (Bodenstein, 1950). In FU-treated larvae, the genital disc underwent no further differentiation after the start of the FU treatment, and the testis never acquired a spiral shape.

The dependence of spermiogenesis on Y-chromosome activity has been investigated by a number of workers (Hess and Meyer, 1968; Kiefer, 1973). Genetic activity of the Y-chromosome, indicated by the formation of DNA loops engaged in RNA synthesis is restricted to the primary spermatocyte stage. Half of the secondary spermatocytes and therefore half of the sperms contain no Y-chromosome. The RNA produced by the Y-chromosome has been shown to be specific (Hess and Meyer, 1968) for spermiogenesis.

The extensive RNA synthesis on Y-chromosomal loops during the long gorwth phase (about 120 hours) of primary spermatocytes has been investigated (Olivieri and Olivieri, 1965; Henning, 1968). On the basis of DNA-RNA hybridization studies, Henning (1968) concluded that Y-chromosomal loops in Drosophila hydei are engaged in synthesis as well as storage of RNA. No RNA synthesis could be detected after meiosis (Olivieri and Olivieri, 1965; Henning, 1968), although extensive protein synthesis was required for spermiogenesis (Brink, 1968). The entire process of

spermiogenesis was believed to be under the direction of stored, long-lived informational RNA accumulated before meiosis (Henning, 1968). Long-lived mRNA has been described in other systems (Davidson, 1968).

In light of the above discussion, it is significant that FU treatment which presumably inhibits mainly RNA synthesis in the presence of exogenous thymidine, did not prevent spermatogenesis in Drosophila. Probably RNA synthesis during early growth of primary spermatocytes had already taken place when treatment began, and the information necessary for completing spermatogenesis persisted in the form of stored mRNA.

Larval ovaries and testes respond differently when transplanted to the adult (Bodenstein, 1947). Ovaries from late third instar larvae, when transplanted with or without ring glands were unable to continue their development in adult hosts. Larval testes, however, even from late second instar larvae transplanted to adult hosts, did show maturation of sperms. They did so even in the absence of ring glands, although the presence of ring glands accelerated sperm maturation (Bodenstein, 1947). Bodenstein concluded that the competence to respond to the low hormonal level prevailing in the adult environment was achieved by larval testis but not by larval ovaries. Maturation of oocytes in ovary in Drosophila is shown to be under hormonal control (King, 1970), but it is not certain whether sperm maturation is also controlled by hormones.

Autonomy in the differentiation of sperms in the testis may be the reason why 5-FU proved to be an effective chemosterilant in screw worm fly females, whereas males were not affected (Crystal, 1963). In Drosophila melanogaster adult females resulting from 8 hour FU-treatment during the third instar stage, show a high degree of sterility; but after a week of being on normal medium, they begin to lay normal viable eggs (Rizki, personal communication).

Imaginal discs

It has been shown by in vitro and transplantation experiments that in Drosophila differentiation of imaginal discs during pupation is under the control of hormones, especially ecdysone (Bodenstein, 1943; Marks, 1970; Mandaron, 1970; Fristrom, 1969). The discs have to attain a state of competence before responding to a hormonal stimulus (Bodenstein, 1943).

The relations between hormones and RNA and protein synthesis in the imaginal discs of various insects have been reviewed by Fristrom (1972). An increase in incorporation of labelled precursors into RNA following injection of α-ecdysone was observed in the wing disc of the cecropia pupa by Wyatt and Wyatt (1971). Injection of ecdysone into Ricini silk worms, particularly when in situ ecdysone titers were low, caused increased incorporation of precursor into RNA during subsequent in vitro incubation of wing discs (Patel and Madhavan, 1969). Exposure of Drosophila imaginal discs in vitro to β-ecdysone caused

increased incorporation of labelled precursors into RNA (Fristrom et al., 1968; Raikow and Fristrom, 1971).

In the present study, FU treatment started at the age of 66 hours or 80 hours prevented the differentiation of imaginal discs. That imaginal discs of FU-treated larvae are not irreversibly damaged has been shown by the transplantation experiments of Rizki (1968). Imaginal discs from larvae following four days of treatment with FU, beginning at the age of 65 hours, underwent metamorphosis when transplanted into normal mature larvae. It is not known whether the failure of the imaginal discs to differentiate in FU-treated larva is due to lack of hormones or to a direct effect of FU on synthesis of RNA required for normal differentiation or some other effect of FU.

Electrophoretic studies

Proteolytic components in the larval homogenate

An analysis of proteolytic digestive enzymes in Drosophila melanogaster during the period from the second day of larval life to adult eclosion was carried out by Waldner-Stiefemeier (1967). When azocasein was used as substrate, maximum activity of digestive enzymes was found in the alkaline range of pH 8.3 at 37°C. Proteolytic activity of the larva increased rapidly during the growth period of the larva but fell off shortly before puparium formation. Activity declined during the pupal stage and rose again in the adult. From results with various synthetic substrates, Waldner-Stiefemeier (1967) concluded that at least three components contribute to the total proteolytic activity in the midgut of Drosophila.

In the present study, it was found that whole homogenates of Drosophila larvae, from the time of hatching to shortly before puparium formation, contained proteolytic enzyme(s) which was activated by incubation of the homogenate in the presence of 1% SDS at 25°C or 37°C for 1 hour. This enzyme was found to be localized in the digestive tract of the larva. Pupal homogenates had no proteolytic activity under similar conditions. These results, in general, confirm the findings of Waldner-Stiefemeier (1967) described above.

The discovery that proteolytic enzyme is activated in vitro by SDS leads to speculation about localization of the enzyme. The proteolytic enzyme is probably contained in

membrane-bound organelles such as lysosomes. The detergent SDS may exert its effect by disrupting the lysosomal membrane and thereby releasing proteolytic enzyme. It was noted that the supernatant of a homogenate centrifuged for 35 minutes at 37,000 g also had proteolytic activity in the presence of SDS. Since lysosomes are presumably centrifuged into the pellet during centrifugation of this magnitude and duration, it may be that proteolytic enzyme is also contained in the membrane bound peroxisomes which would still be in the supernatant and subject to disruption by SDS. It is possible that the proteolytic digestive enzyme of the Drosophila larva is in an inactive form within digestive tract cells and that partial proteolysis or partial denaturation by SDS activates the enzyme. The precise role of SDS in activating proteolytic enzyme(s) in larval homogenates of Drosophila is unknown at this time.

Proteins and isozymes of FU-blocked larva

In general, development is the product of differential gene activity. The qualitative and quantitative changes of the gene products, RNA and proteins, during development are indicators of differential gene activities. Most studies of insect metamorphosis have been conducted with the assumption that larval and adult development involve activity of two different sets of genes. The larval protein spectrum has been used by many workers as a basis of comparison with that of the pupa or adult in documenting the extent of changeover in protein complement during

metamorphosis. Stage-specific and tissue-specific proteins have been studied in various insects including Drosophila (Duke and Pantelouris, 1963; Roberts, 1971, 1975; Pasteur and Kastristis, 1971; Patel, 1971; Thomson, 1975; Chen, 1971; Dickinson and Sullivan, 1975).

Electrophoretic study using SDS gels reveals that the homogenate of early third instar larva has 52 protein bands and one additional band develops during late third instar stage. The homogenate of FU-blocked larva yielded 52 bands at the start of the treatment and as it passed the physical age of late third instar stage, developed the additional band. A new protein band appeared on the gels having pupal homogenate (1 - 4-day-old pupa). Homogenates of FU-blocked larva (up to 10 days in FU treatment) did not develop this pupal specific band. Fluorouracil treatment, therefore, not only prevented the morphological change-over from larva to pupa, but also inhibited the biochemical changeover represented by the development of pupal specific protein.

It is surprising to note that there is a difference of only one protein band during a period when massive degeneration of several larval tissues and differentiation of imaginal tissues occur. However, extreme caution should be taken when interpreting differential gene activity based on differences in electrophoretic patterns of proteins. Some proteins may be present at levels below the level of detection by the method used. The appearance of a protein

at a specific stage does not necessarily mean that the gene was read out just prior to the time when the protein was made. Post-transcriptional controls are known to exist so that some mRNA's are stable for a long time. The continued presence of a protein may not reflect the continued activity of the gene. Slow utilization, during pupal stages, of reserve proteins which were synthesized and stored during larval stages has been demonstrated (Thomson, 1975; Chen, 1971). Minor quantitative differences, which may have major significance in ontogenetic changes, may be masked by inherent problems in methods of detecting and measuring proteins.

Protein synthesis during metamorphosis of Dipteran insects is a controversial subject. Based on the studies of lysine pool size and its turn-over rate during metamorphosis of the blowfly, Lucilia, Williams and Birt (1972) concluded that the formation of proteins in adult insect occurs by de novo protein synthesis during metamorphosis. In a similar study of the amino acid pool and the study of hemolymph proteins in Phormia, Chen and Levenbook (1966) have concluded that there is no massive alteration of larval proteins during metamorphosis. Chen (1971) further speculates that there is no switchover from a larval set of genes to an adult set of genes. Insect development in his view, is a continuous process in which the larval period is a feeding and synthetic period when proteins are synthesized and stored (e.g., the fat body

reserves) to be utilized by differentiating imaginal tissues. Instead of the breakdown of these reserve proteins to the amino acid level and the resynthesis of new proteins during differentiation of imaginal tissues, Chen envisions larval reserve proteins which are probably reutilized at a polypeptide level (Chen, 1971). In the present study, there are no differences in the general protein patterns of 1-to 4-day-old pupae, during a period when differentiation of adult structures and breakdown of some larval tissues is occurring.

Many of the earlier studies that describe the differences between larval and pupal stages based on electrophoretic pattern of proteins and isozymes (Chen, 1971; Wright and Shaw, 1970; Pasteur and Kastritsis, 1971), are open to question in the light of the fact that there is proteolytic activity in the larval homogenate. The differences in protein and isozyme pattern between larva and pupa may be artifacts produced by the presence of proteolytic enzyme(s) in larval homogenate and its absence in pupal homogenate. Hence, reconsideration of the results from the experiments of earlier workers becomes more important in the discussion of ontogenetic changes of proteins.

The significance of the changing isozyme patterns of α- and β-esterases, acid and alkaline phosphatases during Drosophila development has been considered by several workers (review by Dickinson and Sullivan, 1975).

In the present study it was found that isozyme patterns of the four enzymes studied were different in homogenates from the larva and the pupa, and that FU-blocked larval homogenates yielded only the larval pattern of isozymes.

Acid phosphatases are often considered as lytic enzymes and mainly contained in the lysosomes. The pupal specific isozyme pattern of this enzyme may be important for the histolysis of the larval adipose tissue and salivary glands, which normally occurs after puparium formation. Fluorouracil-blocked larvae showed only the larval pattern of this isozymes and not the pupal pattern. This may be the reason why histolysis of larval adipose tissue and salivary glands is prevented from occurring in the FU-blocked larva.

On the whole, the FU-blocked larva continues to display the larval characteristic pattern of proteins and specific isozymes. In conclusion, this study indicates that intracellular biochemical events and the patterns of protein synthesis remain coupled with the stages of differentiation rather than the absolute time of survival of the animal.

Summary

Administration of 5-fluorouracil to mid-third instar larvae of Drosophila melanogaster, 65 or 80 hours of age, (at 25^{o}C) results in blocking the puparium formation and subsequent development. This effect of FU can not be overcome by simultaneous addition of thymidine and/or uridine. The inhibitory effect of FU on Drosophila

development may not be solely at the level of DNA synthesis.

The FU-block is reversible if larvae treated in FU for as long as 72 hours are transferred to regular food medium. Larvae thus transferred go through normal pupation, but there is a very long lag period before they form the puparium.

Histochemical studies reveal that the imaginal discs do not undergo any development and differentiation in FU-blocked larva. The larval adipose tissue and salivary glands are prevented from developing proteinaceous globules and glue proteins respectively if FU treatment is started at the age of 65 hours, whereas starting the treatment at the age of 80 hours does not block these processes.

Larval adipose tissue and salivary glands undergo histolysis during the pupal stage under normal conditions. But they fail to do so in the FU-blocked larva (65 or 80 hours at the start of treatment), even when the larva has passed the physical age of the adult. It has been demonstrated by several workers that the histolysis of larval tissue is under the control of hormone, secreted by the ring gland. The mechanisms underlying the specific responses of the larval tissues to the hormone may involve changes in the cells' genomic activity. The question still remains whether the failure of histolysis of the larval tissues in FU-treated larva is due to FU's effect at the level of stimulus, namely synthesis and secretion of

hormone, or due to its effect(s) on responding cells.

Fluorouracil blocks the development of ovaries but not the testes. The differentiation of spermatocytes into spermatozoa which normally occurs during pupal stage, occurs in the FU-blocked larva. It is suggested that the failure of FU to block spermatid differentiation may be due to the fact that new RNA synthesis does not take place during sperm differentiation and the protein synthesis that is important at this stage is taking place on long-lived mRNA which might have been synthesized and stored before the FU treatment started. Also, high titer of hormone may not be necessary for the differentiation of sperm.

The electrophoretic experiments reveal that homogenates of mid third instar larvae had 52 protein bands, and those of late third instar larva developed one more additional band. Fluorouracil treatment, started at 65 or 80 hours of age, does not block the development of the late larval protein. Homogenate of pupa in the control yields one more protein band, totalling 54 protein bands on SDS acrylamide gels. This pupal specific band is never developed in FU-blocked larva.

Isozyme studies for α- and β-esterases, acid and alkaline phosphatases show that FU blocks the development of pupal specific patterns of these isozymes in the FU-blocked larva.

In conclusion, this study indicates that biochemical events at the intracellular levels and the pattern of protein synthesis remain coupled with stages of development and differentiation rather than absolute time of survival of the animal.

Conclusions

1. Fluorouracil blocks the development and differentiation of the imaginal discs as well as the metamorphosis and histolysis of larval tissues.
2. Early blocking of the developmental processes fails to block the late events of larval life.
3. The fluorouracil effect in blocking the puparium formation is reversible if the treated larva is transferred to food.
4. The isozyme pattern of early and late larval periods of the third instar stage was not blocked by FU treatment in this study; but the pupal isozyme pattern was successfully blocked.
5. Analysis of the protein pattern of FU-blocked larvae by SDS acrylamide gel electrophoresis shows that there are 53 larval bands. One additional band which is specific to pupal stage never appears in the FU-blocked larva.

APPENDIX I

Cream of wheat medium

4500.0 ml water
607.5 ml molasses
562.5 g cream of wheat
22.5 ml tegosept

These amounts are sufficient for the preparation of 52 dishes each containing approximately 50 ml of medium.

Cornmeal - molasses medium

4910.0 ml water
41.0 g shredded agar
412.0 ml molasses
550.0 g cornmeal
47.0 g Brewer's yeast
33.0 ml propionic acid

These amounts are sufficient for the preparation of 70 half-pint bottles each containing approximately 75 ml of medium.

APPENDIX II

Stock solutions for SDS polyacrylamide gels

All stock solutions are made with glass distilled water, filtered through Whatman #1 filter paper and stored in dark bottles in the refrigerator. Catalyst stock and electrode buffer are made fresh for each run. Solutions are brought to room temperature before use. After combining stocks A and C for separating gel, solution is deaerated by suction.

Stock A

Tris	13.6 gm
1 N HCl	18.0 ml
TEMED	0.075 ml
SDS	0.3 gm
Distilled H_2O	to 100 ml

pH = 8.8

Stock B

Tris	4.4 gm
1 N HCl	36.0 ml
TEMED	0.075 ml
SDS	0.3 gm
Distilled H_2O	to 100 ml

pH = 6.8

Stock C

Acrylamide	30.0 gm
Bis	0.8 gm
Distilled H_2O	to 100 ml

Stock D

Acrylamide	9.0 gm
Bis	0.27 gm
Distilled H_2O	to 100 ml

Catalyst Stock

Ammonium persulfate 0.1 gm/100 ml distilled H_2O

Separating Gel (10% acrylamide)

1 part Stock A
1 part Stock C
1 part Catalyst Stock

Stacking Gel (3% acrylamide)

1 part Stock B
1 part Stock D
1 part Catalyst Stock

Electrode Buffer

Tris	3.03 gm
Glycine	14.40 gm
SDS	1.00 gm
Distilled H_2O	to 1000 ml

pH = 8.3

APPENDIX III

Stock solutions for 6% polyacrylamide gels

All the stock solutions are made with glass distilled water, filtered through Whatman #1 filter paper and stored in dark bottles in the refrigerator. Solutions are brought to room temperature before use.

Stock A

Tris ....13.6 grams
1N HCl ..18 ml
TEMED ... 0.075 ml
H_2O to make 100 ml
pH = 8.8

Stock B

Tris .... 4.4 grams
1N HCl ..36 ml
TEMED ... 0.075 ml
H_2O to make 100 ml
pH = 6.6

Stock C

Polyacrylamide .. 18 grams
Bis ..... 0.48 grams
H_2O to make 100 ml

Stock D

Polyacrylamide .. 9 grams
Bis ..... 0.27 gram
H_2O to make 100 ml

Catalyst Stock

0.1 gram ammonium persulfate per 100 ml of H_2O.
Made fresh before use.

Electrode Buffer

Tris 3.03 grams
Glycine 14.4 grams
H_2O to make one litre
pH = 8.3
Made fresh for every run

Separating Gel (6% acrylamide)

1 part Stock A
1 part Stock C
1 part Catalyst Stock

Stacking Gel (3% acrylamide)

1 part Stock B
1 part Stock D
1 part Catalyst Stock

APPENDIX IV

acrylamide: Canalco

α-naphthyl acetate: Sigma

α-naphthyl phosphate: Sigma

β-naphthyl acetate: Sigma

bovine serum albumin: Armour Pharmaceutical Company

bromphenol blue: Allied Chemical

chymotrypsinogen: Sigma

Coomassie blue: Canalco

fast GBC garnet salt: Sigma

fast red TR: Sigma

5-fluorouracil: Hoffman-LaRoche, Inc.

lysozyme: Sigma

2-mercaptoethanol: Eastman Organic Chemicals

mercuric chloride: Mallinkrodt

N, N' methylenebisacrylamide H. P.: Canalco

myoglobin: Sigma

ovalbumin: Sigma

sodium dodecyl sulfate: BDH Chemicals Ltd.

soy bean trypsin inhibitor: Sigma

N, N, N', N', tetramethylenediamine H. P.: Canalco

thymidine: Sigma

tris (Trizma base): Sigma

uridine: Sigma

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